Vol. XXVI, No. 6 1932

MAR 13 1933

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

EDITORIAL COMMITTEE

PROF. G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
SIR F. G. HOPKINS
SIR F. KEEBLE
PROF. W. RAMSDEN
SIR E. J. RUSSELL

LONDON:
CAMBRIDGE UNIVERSITY PRESS
Fetter Lane, E.C.4
also
CHICAGO: The University of Chicago Press
(Agent for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan
TOKYO: Maruzen Company, Ltd.

All rights reserved

1932

Price Fifteen Shillings net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

Members are entitled to receive a copy of the Journal. Further information as to the conditions of membership may be obtained on application to the Hon. Sec., Prof. A. C. Chibnall, Imperial College of Science and Technology, South Kensington, S.W. 7, or to the Hon. Treas., Mr J. Addyman Gardner, 12B Hyde Park Mansions, London, N.W. 1, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. A. Harden, F.R.S., Sunnyholme, Bourne End, Bucks. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3. 10*s*. 0*d*. net per volume (post free) payable in advance) should be addressed to The Cambridge University Press, Fetter Lane, London, E.C. 4, or to the Hon. Treas., Mr J. Addyman Gardner, 12B Hyde Park Mansions, London, N.W. 1. For prices of back numbers and volumes see list on p. 4 of Wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America and has authorised the following subscription rate, viz. $17.00.

Notice to Contributors.

1. Contributors who are members of the Biochemical Society receive 25 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned. Other contributors may purchase reprints but receive no free copies.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912), *Z. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards or ordinary curve paper in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

MSS. for the successive numbers of Vol. 27 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICES.

ALFRED CHASTON CHAPMAN.

1869–1932.

In the passing of Alfred Chaston Chapman on October 17th, in his 63rd year, we have to record the loss of one who, by reason of his striking personality no less than of his scientific attainments, occupied a conspicuous position in the chemical world, and of one who enjoyed an international reputation more particularly in relation to the industrial aspects of fermentation.

He was the son of the late A. W. Chapman, formerly of Pool-in-Wharfedale and of Leeds, and was educated at the Leeds Grammar School, proceeding later to University College, London, where he studied under Williamson, Charles Graham and Carey Foster. Here at an early age he was promoted to the post of "demonstrator of chemical technology" in Graham's laboratory, and as Graham devoted the greater part of his technical activity to matters relating to brewing, Chapman's attention became early focused in this direction, with the result that, after a short period as Graham's private assistant, he was led, under encouragement from his teacher, to establish a consulting practice of his own in connection with which for many years he conducted a private school of brewing in which subject he soon became a recognised authority.

His interests, however, rapidly extended in other directions and there stands to his credit a long list of valuable investigations on multifarious subjects. Most of his contributions to our literature were published in the *Journal of the Chemical Society*, the *Analyst* and the *Journal of the Institute of Brewing*, and have been dealt with in a longer memoir which the writer, at the request of its Council, has contributed to the *Journal of the Chemical Society*; it may suffice here to direct attention more particularly to a brief record of his contributions coming more especially within the general purview of biochemistry.

His first conspicuous effort in this direction was his classical investigation on the essential oil of hops, of which he demonstrated that the principal constituent was the sesquiterpene to which he gave the name "humulene," from which he prepared a number of derivatives; he also isolated the constituent myrcene, identical with that prepared by Power from bay oil, while minor constituents of the lower boiling-point fractions were found to be linalool and linalyl *iso*nonoate, together with some less perfectly identified bodies. Among the higher-boiling fractions he found three previously unrecognised

constituents, namely, a ketone (luparone), an unsaturated alcohol (luparenol), and a phenolic ether (luparol), and he also recognised the presence of *iso*valeric acid (*J. Chem. Soc.* 1895, 780; 1903, 505; 1928, 785 and 1304).

The nitrogenous constituents of hops were also the subject of investigation by Chapman (*J. Chem. Soc.* 1914, 1895) who, among these, identified betaine, *l*-asparagine, aspartic acid, adenine, hypoxanthine and (probably) traces of coniine. Incidentally it may be mentioned that he devoted a good deal of attention to other essential oils in the analysis of some of which he became recognised as an expert.

He also carefully investigated the changes undergone by hops during storage and the assessment of their preservative effects by biological methods, as well as by analysis; his work in this direction, as on many other problems of the brewing industry, is recorded in the volumes of the *Journal of the Institute of Brewing* ranging over a period of nearly forty years.

In 1916 a sample supposed to be cod-liver oil, which found its way to his laboratory, was found to contain nearly 90 % of a hydrocarbon oil which appeared at first sight to be the result of a gross adulteration with mineral oil; but enquiry indicated that, although spurious in the sense of not being cod-liver oil, this remarkable sample was really a genuine product of the livers of certain fish, namely, two Mediterranean species of the shark family, *Centrophorus granulosis* and *Scymnus lichia*. Chapman arranged that a supply of the livers of these fish should be submitted to him from which he extracted an abundance of oil similar to the sample originally examined, containing about 90 % of hydrocarbon oil. To this remarkable hydrocarbon he gave the name "spinacene," describing it as an optically inactive chain compound having the empirical formula $C_{30}H_{50}$ and yielding the saturated compound $C_{30}H_{62}$. By a curious coincidence, unknown to Chapman until he had presented his original paper to the Chemical Society (*J. Chem. Soc.* 1917, 56), a similar hydrocarbon had been independently isolated in Japan by M. Tsujimoto from two other fish of the same family, to which the discoverer had assigned a similar formula and for which he had proposed the name "squalene." Chapman continued his investigation of spinacene (*J. Chem. Soc.* 1918, 458 and 1923, 769), making a closer study of its physical constants and preparing further derivatives, concluding that a more correct empirical formula was $C_{29}H_{48}$, and that it bore a close relation to the terpenes and apparently to cholesterol, which was interesting in view of its origin.

Another memorable biological paper was communicated in 1926 to the Society of Public Analysts (*Analyst*, 1926, **51**, 548) on "The Presence of Compounds of Arsenic in Marine Crustaceans and Shell Fish." Chapman found in the wet flesh of oysters from 10 to more than 30 parts per million of arsenic, calculated as arsenious oxide, while (similarly calculated on the wet edible portion) he found in escallops 85 parts per million, in mussels 100 parts per million, in lobsters from 35 to 100 parts per million, in crabs 36 to 70 parts per million, in shrimps and prawns from 17 to 174 parts

per million, and similar quantities in crawfish, cockles and periwinkles. That the arsenic was in organic combination was sufficiently indicated, but the form of combination still awaits elucidation. The presence of very appreciable traces of arsenic in fish had been previously demonstrated during the work of a Swedish Government Commission and by H. E. Cox in England, and its presence in oysters had been shown in our own Government laboratory in an investigation made for the Ministry of Agriculture and Fisheries; but Chapman's results were by comparison startling, raising the question as to whether these relatively heavy quantities of arsenic, in whatsoever combination it might exist, might not have a bearing on the well-known idiosyncratic susceptibility of some people to distressing cutaneous symptoms as a sequel to the consumption of lobsters or shellfish. Fresh-water crayfish and molluscs were found to be practically free from arsenic.

Chapman was the author, in conjunction with his assistant, Mr F. G. S. Baker, of an "Atlas of the *Saccharomycetes*," published by the *Brewing Trade Review*, 1905, containing more than 100 beautifully executed photomicrographs with descriptive notes. In 1920 he delivered the Cantor lectures before the Royal Society of Arts on "Micro-organisms and some of their Industrial Uses." In these lectures he pleaded for the foundation of a National Institute for Industrial Microbiology, and further emphasised the need of this in his Presidential Address to the Royal Microscopical Society in 1926.

In addition to this presidency, he had been President of the Society of Public Analysts, of the Institute of Chemistry and of the Institute of Brewing; and he was a member of the Royal Commission on Awards to Inventors, of the Scientific Panel of the Board of Trade under the Safeguarding of Industries Act, of the Forest Products Research Board of the Department of Scientific and Industrial Research, and of the Advisory Committee on Plant and Animal Products of the Imperial Institute. The Fellowship of the Royal Society was conferred upon him in 1920, and he was an Honorary Member of the Société de Zymologie of Belgium, and an Honorary Professor of the École Supérieur de Brasserie of Ghent.

The versatility of his chemical interests and his love of mingling socially with his chemical colleagues, his general amiability and his kindly readiness in extending advice or assistance to others, will make his loss at a comparatively early age keenly felt in a wide circle of friends at home and abroad, many of whom will extend their sympathy to Mrs Chapman (the daughter of his old friend, Mr John Astleford of Bournemouth), to whom he was married in 1906 and who has for many years played a pleasant part in social chemical gatherings.

B. D.

SIDNEY JAMES BUCHANAN CONNELL.

1900–1932.

S. J. B. Connell, whose death occurred in South Africa in 1932, was born in 1900. He was educated at Charterhouse and won an Exhibition to University College, London, where he graduated B.Sc. in 1921. In the following year he entered Prof. Drummond's research laboratory where he worked on the form of sulphur in proteins and on the estimation of lipochromes. In 1923 he went as assistant to Dr Zilva at the Lister Institute and collaborated in two papers on the antiscorbutic vitamin published during 1924. Always delicate in health he was obliged to leave England in 1924 and took up farming in South Africa, where he remained until his death.

Connell had an original turn of mind and great ingenuity in devising apparatus for special purposes. He made many friends at University College and the Lister, by whom his early death is greatly deplored.

A. H.

MOSES JOHN ROWLANDS.

1877–1932.

M. J. Rowlands began his medical training at University College Hospital, London, and later studied at Brussels and Berlin. On his return to London he established a practice as a consultant which, except for a period during the War when he acted as Pathologist at Military Hospitals at Mile End and Netley, he maintained until a few years ago when he decided to devote his whole time to other interests he had built up.

His early life had given him a deep love of the country and he farmed a considerable acreage of land between London and the South Coast. To this estate he would escape whenever he could and spend his time from morning to night superintending the activities there. He became a highly successful breeder of pedigree pigs and spent a great deal of time and money in carrying out investigations on problems of practical animal feeding. His wide experience in this field gained for him a considerable reputation, and his writings and lectures never failed to arouse interest in agricultural circles. He did much to draw the attention of the farmer to the need for improving the "vitamin-balance" of rations. His interests in this direction grew so strong latterly that he retired from medical practice to devote himself to the commercial production of balanced food mixtures for animal consumption.

From his youth he had shown a forceful personality and the possession of apparently inexhaustible energy. In all that he did this amazing vigour and determination were revealed. His enthusiasm for original investigation was

real and he spent most of his scanty free time at the bench or making experiments at his farm. Much of his work has not been published, but his printed papers include valuable contributions to the study of rheumatoid arthritis and certain deficiency disorders in man and animals.

All who met him admired his amazing capacity for work and his boundless enthusiasm; those who made his circle of intimate friends knew him for an open-hearted and affectionate Celt. Many of us could see that he habitually overworked and that inevitably a breakdown would occur if he did not take reasonable care of his health. Rest he would not and early this year the warning came in serious form. For a time he took things quietly, but he could not remain inactive and he was soon back at work; within a week or two his condition became grave and on the 22nd of June he died.

J. C. D.

CCIII. THE SUGARS OF URINE.

I. DETERMINATION OF THE REDUCING SUGARS OF URINE.

By EDWARD STAUNTON WEST
and VERNON LESLIE PETERSON.

From the Laboratory of Biological Chemistry, Washington University School of Medicine, Saint Louis, Missouri.

(*Received August 2nd, 1932.*)

Harding and Selby [1931] have recently published extensive observations on the output of fermentable sugar in normal urine. Their report prompts us to record some findings made in this laboratory during recent years. Our plan has been to study the daily excretion of both fermentable and non-fermentable sugar by normal individuals and with these data for comparison to investigate various factors which influence the excretion.

As will be noted from the introduction of the paper by Harding and Selby the question of a suitable method of analysis is paramount in an investigation of this kind. This is especially true when determination of non-fermentable as well as fermentable sugar is involved. Here the efficient pre-treatment of the urine to remove non-sugar reducing substances is imperative. Precipitation with mercuric nitrate-sodium bicarbonate according to Benedict and Osterberg [1918] is probably the most efficient treatment which has been reported in the past, yet this method has the disadvantage of leaving a high salt concentration in the filtrate and there is danger of adding too much bicarbonate with resulting oxidation of sugar by the mercuric ion. Treatment with Lloyd's reagent which was introduced by Folin and Berglund [1922] and which has been used in various ways by different workers, according to our experience, leaves much non-sugar reducing material. In order that maximum accuracy be obtained it is also advisable to use a sugar method capable of estimating small quantities.

Preliminary to their studies, the writers have developed a procedure of analysis for fermentable and non-fermentable sugar in urine which seems to them to represent an improvement over former methods. It is based upon three procedures. Filtrates of urine are prepared by the $HgSO_4$-$BaCO_3$ technique of West, Scharles and Peterson [1929] which has been found effective in removing interfering nitrogenous substances from biological fluids without introducing appreciable amounts of electrolytes and without loss of sugar.

The reducing substances are determined in these filtrates before and after yeast fermentation, using modified Shaffer-Hartmann copper reagents, made much more sensitive than the original due mainly to omission (or decrease) of KI. The reagents used are the outgrowth of unpublished work by Drs Shaffer and Somogyi. The suitability of such reagents for the determination of low concentrations of sugar has been shown also by Harding and Downs [1929], Harding and Van Nostrand [1930] and Harding and Selby. Yeast fermentation is carried out with washed yeast prepared according to Somogyi's [1927] directions. We believe that this combination gives more nearly correct reducing values for fermentable and non-nitrogenous non-fermentable reducing substances (possibly of carbohydrate nature), than has been achieved by other procedures.

This paper reports in detail the analytical methods used and the daily excretion of fermentable and non-fermentable sugars in the urines of a number of normal human subjects. A second paper has to do with some of the factors which influence the excretion of these substances.

Reagents.

Mercuric sulphate. A saturated solution in 10 % H_2SO_4 prepared by dissolving 27 g. $HgSO_4$ per 100 cc. of cold acid (5–10°). The solution is decanted from the basic sulphate which separates as the solution comes to room temperature.

Barium carbonate[1]. Mallinckrodt's technical precipitated $BaCO_3$, dried.

Yeast. Fleischmann's yeast washed and kept as recommended by Somogyi.

Sugar reagent. Modified Shaffer-Hartmann containing 1 g. KI per litre, kept in pyrex flasks[2].

Urine. Urine samples were preserved with a little toluene and kept in the cold room.

Procedure of analysis.

Preparation of filtrates. 10 cc. of urine are pipetted into a 500 cc. Erlenmeyer flask, together with 45 cc. of water, and 15 cc. of mercuric sulphate reagent are added from a burette. To the mixture are added 35–40 g. of $BaCO_3$ with vigorous rotation for a few seconds until most of the CO_2 has escaped. The flask is tightly closed with a rubber stopper, shaken hard and the pressure released. This is repeated until no more pressure develops. If the mixture reddens blue litmus, a little more $BaCO_3$ is added and the shaking repeated. After neutralisation the contents of the flask are poured upon a rapid filter and the filtrate (about 25 cc.) acidified with 1 large drop of conc. H_2SO_4.

[1] One or two samples of $BaCO_3$ have been found to contain something which inhibits the action of yeast. The interfering substance is not removed by H_2S in acid solution.

[2] Details of a number of improved reagents will be published shortly in a joint paper by Drs Shaffer and Somogyi. The reagent used by the writers had the following composition in g. per litre: Na_2CO_3 (anhydrous), 25·0; $NaHCO_3$, 20·0; Rochelle salt, 25·0; $CuSO_4$, $5H_2O$, 7·5; KI, 1·0; KIO_3, 20·0 cc. of *N* sol. per litre.

The filtrate, in a 300 cc. Erlenmeyer flask, is treated with H_2S for several seconds and the H_2S removed by a rapid current of moist air. The precipitated HgS and $BaSO_4$ are filtered off on a small paper and the filtrate received in a large test-tube. The funnels and tubes used must not be contaminated by previous use with solutions containing mercury salts. It is convenient to keep a supply of funnels and tubes especially for this filtration to prevent poisoning the yeast with Hg-contaminated vessels. The time required for the preparation of filtrates is only a little greater than that consumed in making Folin-Wu blood-filtrates. The filtrations proceed rapidly. The urine-filtrates so obtained represent a dilution of 1 : 7. Greater concentration cannot well be achieved. Dilutions up to 1 : 12 give quite satisfactory results when the urines themselves are not too dilute.

Determination of reducing substances. The filtrate obtained by the above method is divided into two approximately equal portions (12–14 cc. each). About 7 cc. of a 15 % suspension of washed yeast are placed in a 15 cc. pointed centrifuge-tube and the yeast centrifuged down firmly. Supernatant liquid is poured off and the walls of the tube dried with a roll of filter-paper. One portion of filtrate is added to the yeast, mixed and allowed to ferment for 10 minutes at room temperature, the yeast being occasionally stirred. After centrifuging the yeast the fermented and unfermented filtrates are analysed for reducing substances as follows. 5 cc. portions, in duplicate, are measured into 25 × 200 mm. pyrex tubes, a drop of 0·05 % aqueous phenol red added to each, and 0·5 *N* NaOH drop by drop with shaking to the red colour of the indicator. 5 cc. of copper reagent are added, the tubes covered with glass bulbs, and heated in a steady rack in an actively boiling water-bath for 15 minutes. The tubes are then cooled to about 30°, 1 cc. of a solution containing 4 % KI and 5 % potassium oxalate is added followed by 5 cc. of *N* H_2SO_4. Upon acidification the tubes are thoroughly shaken (bulbs still in place), until all Cu_2O is dissolved, and permitted to stand for 10 minutes. They are then rinsed down, including sides of covering bulbs, with a fine stream of H_2O from a wash-bottle (3–5 cc.) and titrated with 0·005 *N* thiosulphate, adding a solution of starch towards the end. Heated blanks are run on distilled water (containing phenol red). The latter does not affect the reduction and improves the end-point of the iodimetric titration for most people.

The method of calculating results will be illustrated by an example. Suppose the sugar reagent used was such that 1 cc. of 0·005 *N* thiosulphate titration was equivalent to 0·113 mg. of glucose. The total concentration of reducing substances (as glucose) in mg. per 100 cc. for a titration difference of 3·10 cc. of 0·005 *N* thiosulphate on the unfermented filtrate would be: $3{\cdot}10 \times 0{\cdot}113 \times 7 \times 20 = 49$. A titration difference of 2·0 cc. on the fermented filtrate would represent: $2{\cdot}0 \times 15{\cdot}8 = 32$ mg./100 cc. total non-fermentable reducing substances (as glucose). $49 - 32 = 17$ mg. per 100 cc. fermentable (as glucose).

Discussion of the method.

Filtrates prepared from ordinary urines by $HgSO_4$-$BaCO_3$ precipitation contain about 2–3 mg. of N per 100 cc. of filtrate so that the N content of samples for analysis amounts to 0·1–0·15 mg. Urea, which does not interfere with the sugar determinations, represents nearly all the nitrogen. Urines from high protein diets may give mercury filtrates containing many times this amount of nitrogen as a result of the great increase in urea. Such filtrates show normal values for both fermentable and non-fermentable substances. Pure urea solutions were treated with $HgSO_4$-$BaCO_3$, and the nitrogen of the filtrates determined. The precipitations were made at a volume of 70 cc. as in the case of urine, the amount of material specified being present. 2·76 mg. of N remained in the filtrate of a solution originally containing 45 mg. of urea-N, 8·26 mg. in one containing 90 mg. and 16·7 mg. in the filtrate of a solution to which 270 mg. of urea-N had been added. Creatinine, creatine and uric acid removal were tested similarly. Using 3·75 mg. of creatinine-N (10·16 mg. creatinine) no detectable nitrogen was present in the filtrate (10 cc. digested). 7·5 and 15 mg. of creatinine-N gave filtrates containing respectively 0·68 and 1·88 mg. of unprecipitated N. Of 4·23, 8·46 and 12·69 mg. of creatine-N in solution precipitated with $HgSO_4$-$BaCO_3$, 0·71, 1·94 and 4·7 mg. respectively were found in the filtrates. In a like manner 0·23 and 0·29 mg. N remained in filtrates of solutions containing originally 2 and 3·6 mg. of uric acid-N respectively. As would be expected, uric acid and creatinine are practically undetectable in $HgSO_4$-$BaCO_3$ filtrates of urine.

It is probable that mercuric sulphate and mercuric nitrate precipitations are about equally efficient as far as the removal of interfering substances is concerned, when $BaCO_3$ is used as a neutralising agent, though the $Hg(NO_3)_2$-$BaCO_3$ filtrates of urine give somewhat higher titration values than do the $HgSO_4$-$BaCO_3$ filtrates, probably owing merely to the influence of their high electrolyte concentration on the oxidising equivalence of the copper reagent. Benedict and Osterberg [1918] reported practically no nitrogen in their $Hg(NO_3)_2$-$NaHCO_3$ filtrates. We have found it impossible to determine the organic nitrogen in such filtrates. In the Kjeldahl process of digestion the nitrous acid formed from the nitrate liberates free N_2 from amino-groups and ammonium salts. We have observed complete loss of N in the analysis of urea and glycine caused by adding nitrate during the digestion. It is probable, however, that the nitrogen contents of the $Hg(NO_3)_2$ and $HgSO_4$ filtrates are of the same order. It should be pointed out that $BaCO_3$ is superior to $NaHCO_3$ as a neutralising agent for $HgSO_4$ not only because it yields filtrates of very low electrolyte content, but also because with it there is no danger of developing local alkalinity within the solution being neutralised with consequent loss of sugar as a result of oxidation by the mercuric ion.

The effectiveness of the $HgSO_4$-$BaCO_3$ treatment in removing interfering uric acid and creatinine from solution and giving filtrates which show true

sugar values was demonstrated in the following way. A mixture containing 90 mg. of urea-N, 3·75 mg. of creatinine-N, and 2 mg. of uric acid-N in a volume of 70 cc. gave, without pre-treatment, reduction equivalent to a total of 4·3 mg. of glucose. 7·0, 3·5 and 1·4 mg. of glucose were added to this mixture and precipitated at a volume of 70 cc. as in the case of urine. By analysis 7·1, 3·6 and 1·66 mg. of fermentable sugar respectively were recovered. Titrations of the fermented filtrates were identical with those of the blanks on water. As has long been known, it is essential to remove creatinine and uric acid from urine in order to obtain accurate reduction values for non-nitrogenous non-fermentable substances. $HgSO_4$-$BaCO_3$ precipitation effectively removes sulphate and phosphate, which may be found advantageous in certain analyses. Glucose added to $HgSO_4$-$BaCO_3$ filtrates is quickly and quantitatively fermented by yeast.

Sugar added to urine is quantitatively recovered by analysis of the $HgSO_4$-$BaCO_3$ filtrates.

We have used varying quantities of yeast for fermentation and failed to note absorption of non-fermentable reducing substances from $HgSO_4$-$BaCO_3$ filtrates of urine. Van Slyke and Hawkins [1929] report absorption from Lloyd's reagent filtrates which were analysed by their ferricyanide-gasometric method. Harding and Van Nostrand [1930] found no absorption from such filtrates using essentially the same sugar reagent as we have employed. The results of Van Slyke and Hawkins are perhaps due to their ferricyanide reagent oxidising yeast-absorbable substances which are unaffected by the copper reagent.

Comparison with Folin's method.

It is conceivable that yeast may remove some iodine-absorbing substance which does not reduce copper and which is not sugar, yet, by the method used, would be calculated as fermentable sugar. Accordingly, it was considered desirable to establish the presence of copper-reducing material, utilisable by yeast, by a method not involving iodimetric titration and also to secure data for a comparison of our method with another. It should be pointed out in this connection that $HgSO_4$-$BaCO_3$ filtrates do not absorb iodine from an acid solution, which in itself suggests the improbability of such an error.

Fermentable and non-fermentable reducing substances were determined on both $HgSO_4$-$BaCO_3$ and Lloyd-permutit filtrates of urine by the sensitive Shaffer-Hartmann reagent and by Folin's [1929] reagent. The Lloyd-permutit filtrates were prepared according to Folin and Svedberg [1926]. Table I summarises the data obtained on 24-hour samples of 11 normal individuals, the fermentable and non-fermentable substances being expressed as mg. per 24-hour sample (glucose reducing equivalent). The first column also includes determinations on diluted (1 : 10; 1 : 15 for very concentrated urine) untreated urines by the Shaffer-Hartmann method for comparison.

It will be observed that the average non-fermentable reducing material was 1246 mg. (as glucose) in untreated urine, 581 mg. in Lloyd-filtrates, and

Table I. *Fermentable and non-fermentable reducing substances of urine in untreated urine, $HgSO_4$-$BaCO_3$ and Lloyd-permutit filtrates, determined by Shaffer-Hartmann and Folin methods.*

Results expressed in terms of mg. glucose per 24-hour urine.

	Shaffer-Hartmann						Folin			
	Diluted untreated urine		$HgSO_4$-$BaCO_3$		Lloyd		$HgSO_4$-$BaCO_3$		Lloyd	
Sample No.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.	Ferm.	Non-ferm.
1	123	1895	116	370	125	800	61	362	140	425
2	72	1310	66	304	85	560	66	270	60	406
3	55	1005	73	265	80	496	46	111	50	264
4	82	1270	72	387	66	675	59	351	53	492
5	86	770	118	244	121	428	106	210	94	286
6	41	1163	89	240	84	502	91	211	72	300
7	212	722	104	250	135	545	170	148	138	327
8	85	1465	94	278	119	585	105	237	95	353
9	41	1245	91	260	73	545	39	283	47	351
10	176	1682	119	407	130	813	128	382	164	516
11	52	1180	61	202	72	443	78	144	78	278
Min.	41	722	61	202	66	428	39	111	47	264
Max.	212	1895	119	407	135	813	170	382	164	516
Av.	93	1246	91	292	99	581	86	246	90	362

292 in the $HgSO_4$-$BaCO_3$ filtrates, all by the Shaffer-Hartmann method. Lloyd treatment removed 53 % of the total non-fermentable reducing material and $HgSO_4$-$BaCO_3$ treatment removed 76 %. An interesting point is that, whereas the average difference in reduction equivalent for non-fermentable reducing substances in mercury filtrates by the Shaffer-Hartmann and Folin methods is 16 % (292–246) the difference in Lloyd filtrates is 38 % (581–362). This suggests a decided chemical difference between the reducing material not removed by Lloyd treatment but taken out by mercury and that remaining in the mercury filtrates. If the latter is essentially carbohydrate in character, the former is probably not, and Lloyd filtrates are not to be recommended in studies of the non-fermentable sugar of urine.

The average values for fermentable sugar by the Shaffer-Hartmann method were 93 mg. in untreated urine, 91 mg. in mercury filtrates and 99 mg. in Lloyd filtrates. By the Folin method the averages were 86 mg. in mercury and 90 mg. in Lloyd filtrates. The averages are rather close together but it will be noted that the individual results on mercury filtrates by the Shaffer-Hartmann method show less range of variation than those by any of the other combinations. We have found it possible to duplicate our results by this method more satisfactorily also.

Reducing substances in normal human urine.

Determinations of the daily output of fermentable and non-fermentable reducing substances have been made on a large number of medical students and laboratory workers. The maximum output of fermentable in 55 cases

was 596 mg., the minimum 23 mg. and the average 142 mg. per day calculated as glucose. The average concentration was 12 mg. per 100 cc. with an average urine volume of 1216 cc. The diet was not controlled. The amount of fermentable material varies widely yet it is probably always present. Our results agree, in general, with those of Van Slyke and Hawkins, and Harding and Selby in so far as the results can be compared. These workers analysed samples of urine taken at definite periods during the day and after the ingestion of various foods and did not report the results on 24-hour samples. Harding and Selby observed the absence of fermentable sugar from urine after short periods of starvation. By our method we have always found fermentable sugar in starvation urine though in decreased amounts. The failure of Harding and Selby to find fermentable sugar in starvation urines is probably due to the fact that they used more highly diluted urines, a smaller sample for analysis (2 cc. instead of 5 cc.), a somewhat less sensitive sugar reagent and a shorter time of heating than did the writers. To illustrate, assuming a minimum dilution of 1 : 10 as used by these workers (their maximum dilution was 1 : 20), a urine containing 6 mg./100 cc. of glucose would give a titration difference of about 0·08 cc. of 0·005 *N* thiosulphate, and if the urine dilution had been 1 : 20 the titration difference would be only 0·04 cc., very close to their normal error of titration. The same urine analysed by our procedure would give a titration difference of about 0·37 cc. of 0·005 *N* thiosulphate, nearly five times that by the procedure of Harding and Selby using a 1 : 10 dilution. Other points relating to fermentable sugar are considered in the second paper.

The advisability of using a sensitive sugar reagent in detecting the presence of fermentable sugar in urine seems to be borne out by some of our experiments. At first we used the Somogyi modification of the Shaffer-Hartmann reagent [1926] with 10 g. of KI per litre for $HgSO_4$-$BaCO_3$ filtrates. In 20 normal cases 5 showed the absence of fermentable sugar. Using exactly the same technique, with the exception that the sensitive iodide-free sugar reagent was employed, fermentable sugar was found present in the urines of 57 normal individuals in a group of 58. Many urines analysed since this series was run have shown fermentable sugar without exception, including those from fasting subjects.

The maximum output of non-fermentable substances in 58 cases, was 554 mg. (as glucose), the minimum 273 mg., and the average 395 mg. with an average urine volume of 1208 cc. The average concentration was 33 mg. per 100 cc. The results of Van Slyke and Hawkins for non-fermentable substances were much higher, probably owing to the use of Lloyd-filtrates and the more powerfully oxidising ferricyanide reagent. It should be pointed out, however, that a comparison of non-fermentable reducing substances as obtained by different sugar methods, even with the same filtrate is meaningless as long as the nature of the material and its reducing power toward the different reagents are unknown.

SUMMARY.

An improved method for the determination of the fermentable and non-fermentable sugars of urine is presented. It is based upon the preparation of $HgSO_4$-$BaCO_3$ filtrates followed by determination of fermentable and non-fermentable sugar using washed yeast and a sensitive Shaffer-Hartmann reagent.

The writers have found fermentable sugar present in almost all 24-hour samples of normal urine examined, an average of 142 mg. (as glucose) in 55 cases.

The average non-fermentable sugar (calculated as glucose) was 395 mg. in 58 24-hour samples of normal urine.

The writers are indebted to Mr Arthur Brandon for technical assistance.

REFERENCES.

Benedict and Osterberg (1918). *J. Biol. Chem.* **34**, 195.
Folin (1929). *J. Biol. Chem.* **82**, 87.
—— and Berglund (1922). *J. Biol. Chem.* **51**, 209.
—— and Svedberg (1926). *J. Biol. Chem.* **70**, 406.
Harding and Downs (1929). *J. Biol. Chem.* **84**, 335.
—— and Selby (1931). *Biochem. J.* **25**, 1815.
—— and Van Nostrand (1930). *J. Biol. Chem.* **85**, 765.
Somogyi (1926). *J. Biol. Chem.* **70**, 599.
—— (1927). *J. Biol. Chem.* **75**, 33.
Van Slyke and Hawkins (1929). *J. Biol. Chem.* **83**, 51.
West, Scharles and Peterson (1929). *J. Biol. Chem.* **82**, 137.

CCIV. THE SUGARS OF URINE.

II. FACTORS AFFECTING THE EXCRETION OF FERMENTABLE AND NON-FERMENTABLE SUGARS IN URINE.

By EDWARD STAUNTON WEST, ADOLPH CHARLES LANGE and VERNON LESLIE PETERSON.

From the Laboratory of Biological Chemistry, Washington University School of Medicine, Saint Louis, Missouri.

(*Received August 2nd, 1932.*)

The first paper of this series by West and Peterson [1932] outlines an improved procedure for the determination of fermentable and non-fermentable sugars in urine. Although a number of workers have studied the factors which influence the excretion of these substances, the whole question is in a rather confused and unsatisfactory state. This is largely due to the fact that in much of the older work the methods of analysis were inadequate. This applies especially to the fermentation procedures used, which involved the use of unwashed yeast acting for many hours. Under such conditions it is well recognised that the yeast itself may add an amount of reducing material which may completely offset that removed by the yeast, leading to data which indicate the complete absence of fermentable sugar, or bacterial contamination may cause sugar destruction leading to values for fermentable sugar which are too high. Other sources of error lie in the incomplete removal of non-sugar reducing substances from the urine before analysis and the use of sugar methods incapable of detecting small differences.

Benedict and Osterberg [1918] using $Hg(NO_3)_2$-$NaHCO_3$ filtrates of urine and the picrate sugar method, studied the effect of diet and starvation upon the excretion of total sugar in the dog. They found this to be highest on a carbohydrate diet, appreciably decreased on a high protein diet and markedly less during starvation. They noted a maximum excretion 4–5 hours, and a minimum excretion about 15 hours, after feeding. They found the output to be independent of urine volume. These workers with Neuwirth [1918] studied both the fermentable and non-fermentable sugars in the urine of two normal men as affected by diet. They found the total excretion to be greater, in general, for a high carbohydrate than for a high protein diet, both fermentable and non-fermentable fractions being increased. Their fermentable values for all experiments on one individual averaged nearly the same as their non-fermentable, something over 500 mg. (calculated as glucose) per 24 hours. The other subject

showed less, but approximately the same proportion of fermentable and non-fermentable sugar. The large proportion of fermentable sugar was perhaps due to long periods of fermentation with unwashed yeast during which there may have been considerable destruction of sugar by bacteria. They concluded that fermentable sugar is always present in normal urine and advanced the idea of a continued "glycuresis" or passage of blood-sugar into the urine.

Folin and Berglund [1922] studied the total sugar of urine and blood after the ingestion of large quantities of various carbohydrates. In determining urine-sugar they used Lloyd-filtrates and analysed them by the method of Folin and Wu [1920]. Fermentable sugar was not determined. They noted an increase in total sugar (Lloyd-filtrates contain much non-sugar reducing material) when passing from a high protein diet to an ordinary mixed diet containing carbohydrate, but when pure sugars (glucose or its derivatives) were ingested in large amounts there was no increased output. Fructose was found to cause an increase, especially if previously heated.

Folin and Berglund concluded that there is no "glycuresis" normally of blood-sugar into the urine as stated by Benedict and that the sugars of normal urine are essentially composed of unassimilable carbohydrate substances of the food, occurring there either naturally or formed by heating.

Greenwald, Gross and Samet [1924–25] studied the effect of diet upon the excretion of urinary sugar in both the dog and man. They used $Hg(NO_3)_2$-$NaHCO_3$ filtrates and analysed them by four different methods, the two picrate methods of Benedict and Osterberg [1918; 1921], the copper method of Folin and Berglund and that of Shaffer and Hartmann [1920–21]. Fermentable sugar was not determined. From their observations they conclude that the sugars of normal urine are composed of carbohydrates assimilable with difficulty or not at all and of reducing substances (probably pentose) derived from protein of the food and from endogenous sources. They considered at least one-half of the sugars to originate from food protein and endogenous sources. Their experiments tended to show that practically no reducing material arises from the metabolism of nucleic acids or endogenous nuclear metabolism, as shown by absence of increase in urinary sugar after the ingestion of nucleic acids or as the result of turpentine abscesses in the dog. The marked effect of heated carbohydrate material (grape-nuts) in increasing the reducing material of urine was also observed. Later Greenwald, Gross and McGuire [1927] carried out other experiments and used Eagle's [1926–27] fermentation procedure which consisted of incubation with unwashed yeast for 40 minutes. They concluded that while a part of the urinary sugar may arise from heat-altered carbohydrate of food, this fraction is rather small. The opinion expressed in the former paper that pentose derived from protein constitutes a part of the sugar of urine was abandoned. Also they considered the small fraction of fermentable sugar indicated by their data probably to represent a sugar other than glucose. They suggested the idea that some of the urinary sugar may arise from bacterial action in the intestines. No experiments were reported in support of this view.

Höst [1923], using the method of Benedict and Osterberg, studied the excretion of urinary sugar by many human subjects after various diets. He determined fermentable sugar with unwashed yeast acting for 48 hours. He concluded that bread especially contributes to the sugar of urine and that glucose is not present.

The workers cited above have carried out the principal investigations relating to the effect of diet upon urinary sugar. In none of these cases have the methods of analysis employed been capable of giving an accurate estimate of the fermentable and non-fermentable fractions, and in some no attempt at determining fermentable sugar was made. In fact it was a debatable question until recent years as to whether urine contains any fermentable sugar at all. As noted above, Benedict, Osterberg and Neuwirth found considerable quantities; Shaffer and Hartmann [1920–21], using unwashed yeast, failed to observe fermentable sugar in urine, as also did Eagle [1926–27]. After Somogyi [1927] introduced the use of washed yeast for fermentation and the more sensitive sugar reagents had become available, several workers reported the presence of fermentable sugar in urine. Among these were Van Slyke and Hawkins [1929], and Peterson and West [1929]. At the present time, then, some investigators agree that normal urine contains both fermentable and non-fermentable sugar, though there still seems to be no evidence in the literature that the fermentable fraction is glucose. This represents a separate problem which will be considered in the third paper of this series by West and Steiner. Suffice it to say that evidence has been obtained from experiments on the rate of fermentation of isolated sugars of urine in the Warburg apparatus indicating that the fermentable sugar of urine is glucose.

In view of the conflicting opinions arising from this older work the writers have applied their methods of analysis, reported in the first paper, in an attempt to determine more certainly some of the factors influencing the excretion of fermentable and non-fermentable sugars in the urine. Evidently much of the work is repetition of that formerly done by the older methods of analysis. This seemed not only desirable, but necessary, in order that we might continue our studies as to the chemical nature of these sugars.

The factors specifically studied have included the effects of diet, starvation, renal damage, glandular stimulation and constipation.

Factors affecting the excretion of fermentable and non-fermentable sugars.

Diet.

Non-fermentable sugars. A diet entirely free from substances that will contribute to the non-fermentable fraction cannot be realised. Accordingly, a preliminary study was made to discover the foods which, taken in reasonable quantities, have no marked effect upon the excretion of non-fermentable substances. It was found in brief that white bread, milk, potatoes, sucrose

starch, fats, peas, string beans, tomatoes, citrus fruits, canned pineapple and pears, strawberries, bananas, lettuce, cabbage, celery, eggs, cheese, cream of wheat, meats cooked without cereal, and some other articles of food can be taken rather interchangeably in mixed diets with a practically constant elimination of non-fermentable reducing substances.

A number of experiments on different individuals in which the subjects ate mixed diets of these foods on successive days were run. The results on two individuals are illustrative. Over a period of 7 days, A.C.L. excreted daily a maximum of 360 mg. of non-fermentable sugar (as glucose), a minimum of 312 and an average of 335 mg.; a maximum of 136 mg. of fermentable sugar, a minimum of 96 and an average of 117 mg. In the case of E.S.W. over a period of 5 days a maximum of 351 mg. of non-fermentable sugar, a minimum of 312 and an average of 325 mg. were eliminated. The fermentable sugar output showed a maximum of 158 mg. and a minimum of 111 mg. The values can be made to vary appreciably from these by varying the relative proportions of the different foods taken, though it seems that significantly high values cannot be produced by quantities which the average person cares to eat. Urine volume was found to be not detectably related to the excretion of reducing substances, even with extreme diuresis induced by forced fluids. This is in accord with the observations of other workers.

Muscular exercise appears to be unrelated to the output of the non-fermentable fraction as found by experiments on a subject exercising normally and then resting in bed.

In order to determine the effect of the ingestion of large quantities of different proteins, carbohydrates, fruits and other substances, restricted diets of the above foods were employed. The subject was fed with the restricted diet for 2–3 days previous to the administration of the food or substance to be tested and also for 1–2 days thereafter if a positive effect was noted.

A. The following combinations either supplementing or replacing the restricted diet caused no significant increase and in some instances a decrease of the 24-hour excretion of non-fermentable reducing substances: thirty egg-whites; 280 g. of cheese and 12 eggs; 1000 g. of herring roe; 1125 g. of brains (pig); 900 g. of boiled kidney (beef); 900 g. of boiled liver (pig); 675 g. of pancreas (ox); 675 g. of boiled beefsteak; 560 g. of canned sweet corn (Del Monte); 200 g. of dextrin (Merck's white), 100 g. at 10.30 a.m. and 100 g. at 3.30 p.m.; 600 g. of glucose in 4 portions of 150 g. each during the day; 200 g. of starch and 250 g. of sucrose; 14 g. of yeast nucleic acid; 1200 g. of bananas (net); 1350 g. of white seedless grapes.

B. The foods listed below did cause a marked increase in the output of non-fermentable substances: 500 g. of grape-nuts, 936 mg., 125 % increase; 450 g. of blue label Karo syrup (dark), 643 mg., 68 % increase; 125 g. of caramelised sucrose, 534 mg., 68 % increase; 400 g. of honey in 3 portions, 555 mg., 83 % increase; 450 g. of raisins and 280 g. of dates, 884 mg., 115 % increase; 1350 g. of unpeeled apples, 554 mg., 40 % increase.

It will be seen that the non-fermentable sugars are increased by the ingestion of such things as grape-nuts, dark Karo syrup, honey, carmelised sugar, dates, raisins and apples, but unaffected by pure carbohydrates as starch, sucrose, dextrin and glucose. These findings are in accord with the ideas of Folin and Berglund and others that much of the reducing material of normal urine represents unassimilable foreign and altered carbohydrate substances from the diet. Non-fermentable sugars are not increased beyond the values of the restricted diets by high protein intake. It was noted, however, that meat rolled in flour and cooked so as to yield caramelised carbohydrates greatly increases these substances. In the experiments reported, this was avoided, the meat being either boiled or fried plain. The effect of liver may be large if it is fried instead of boiled, presumably owing to alteration of the carbohydrate contained in it. Nucleoprotein diets and nucleic acid (yeast) caused no definite

increase of non-fermentable sugars. These results are in agreement with the findings of Greenwald, Gross and Samet [1924–25].

Non-fermentable sugars have been determined in several of the foods used. In $HgSO_4$-$BaCO_3$ filtrates of thoroughly macerated beef kidney, liver and muscle 9, 60 and 25 mg. (as glucose) per 100 g. of tissue were found respectively. These were increased by hydrolysis of the tissues for 5 hrs. in 0·5 *N* H_2SO_4 to 282 mg. in kidney, 229 in liver and 220 in muscle per 100 g. Karo corn syrup (dark), honey and caramelised cane sugar were found to yield considerable amounts of non-fermentable reducing substances both before and after hydrolysis. Ordinary commercial sucrose also gave appreciable non-fermentable reducing materials upon hydrolysis. Human faeces from a restricted diet were found to give about 20 mg. per 100 g. (as glucose) of non-fermentable reducing substances in $HgSO_4$-$BaCO_3$ filtrates.

It seems likely that practically all foods contribute something to this non-fermentable fraction which is apparently made up of a very heterogeneous group of substances essentially non-nitrogenous, and probably chiefly carbohydrate in nature.

The mercury filtrates of urine from different diets have been hydrolysed for 5 hrs. with 0·5 *N* H_2SO_4 and fermentable and non-fermentable sugars determined. Table I records data obtained and shows that the non-fermentable

Table I. *Effect of hydrolysis for 5 hrs. with 0·5 N H_2SO_4 upon the non-fermentable and fermentable sugars of urine.*

Mercury filtrates of urine				
Non-fermentable sugar total (mg.)		Fermentable sugar total (mg.)		
Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Diet
644	620	146	1684	A.C.L. Restricted + 450 g. dark Karo syrup
316	399	142	198	E.S.W. Restricted
515	545	136	1160	E.S.W. Restricted + 125 g. of caramelised sucrose
885	862	243	256	A.C.L. Restricted + raisins and dates
274	279	68	129	E.S.W. Second day of starvation.
305	354	102	230	E.S.W. 1000 g. herring roe
936	826	72	107	E.S.W. 500 g. grape-nuts

fraction is not, on the average, appreciably increased by hydrolysis and is, for the most part, not of a polysaccharide character. Crystalline osazones (apparently a mixture) have been prepared from fermented mercury filtrates of normal urine, controls being run on the yeast. This material melted at 165–170° (uncorr.) with decomposition at 175°, and seemed to resemble the product

obtained by Patterson [1926]. It is interesting to observe that the fermentable substances in $HgSO_4$-$BaCO_3$ filtrates are appreciably increased by hydrolysis, even during starvation. We have found this to be true in a number of experiments not listed in the table. It should be noted that Folin and Berglund [1922] observed an increase in total sugar of urine after hydrolysis as did Greenwald, Gross and McGuire [1927]. These workers, however, did not determine that the increase is chiefly concerned with the fermentable fraction of the sugars. Since this apparently cannot arise from the non-fermentable fraction, the conclusion seems warranted that diffusible non-reducing polysaccharides are normally excreted. It will be pointed out in the third paper that there is probably very little if any fermentable reducing polysaccharide in urine and, bearing the above facts in mind, it seems that the carbohydrates of both normal and starvation urines may be chiefly grouped into the following three classes:

A	B	C
Non-fermentable simple sugars or derivatives	Non-reducing polysaccharide material	Fermentable sugar
Unchanged by acid hydrolysis	Converted into fermentable sugar by hydrolysis	Glucose

The kidneys must effect a relatively very high concentration of the polysaccharide substances from the blood because Scharles and West [1931] have shown the absence of detectable hydrolysable sugar from tungstic acid blood-filtrates. The same thing is true for the non-fermentable reducing fractions of mercury filtrates.

It has regularly been observed that the ingestion of large quantities of sucrose leads to increased values for fermentable sugar. This can be accounted for in part or entirely by the excretion of unchanged sucrose in the urine, some of which is inverted during the acid mercuric sulphate precipitation used in the preparation of filtrates. Sucrose solutions treated with the mercuric sulphate reagent as in the preparation of urine-filtrates have been shown to be considerably hydrolysed. Values for fermentable sugar after the ingestion of quantities of sucrose sufficient to cause sucrosuria, are, therefore, not significant when the urine is precipitated by a strongly acid reagent such as $HgSO_4$ or $Hg(NO_3)_2$.

Fermentable sugar. The state of activity of the pancreas and general ability of the body to metabolise glucose are recognised as important controlling factors in the elimination of fermentable sugar in the urine. For a discussion of this effect the papers of Greenwald, Gross and Samet [1924–25], and those of Heinbecker [1928] and Dann and Chambers [1930] may be consulted. It is generally recognised that carbohydrate starvation as induced by fasting and to a less degree by protein and fat diets decreases the ability of an animal to metabolise carbohydrates. This condition followed by a carbohydrate meal may lead to a glycosuria which is easily detectable by the usual qualitative sugar tests. This effect on the small quantity of fermentable sugar normally excreted, which would not be recognised as glycosuria by the ordinary tests, seems to be clearly shown in a series of experiments on E.S.W.

On 8. xi. 29, with a restricted diet, the fermentable sugar excretion was 159 mg. The following day the subject fasted and the fermentable sugar dropped to 62 mg. The next day 900 g. of boiled pig-liver constituted the sole diet and the fermentable fraction was 76 mg. The following day an ordinary restricted diet was taken which normally would have caused the excretion of not over 150 mg. of fermentable sugar. Actually 445 mg. were found, about three times the expected amount. The reverse effect seems to be shown in another series on the same subject. On 19. xi. 29, E.S.W. excreted 131 mg. of fermentable sugar on a restricted diet. The next day the restricted diet *plus* 100 g. of sucrose at breakfast, 50 g. at lunch and 100 g. at dinner was taken, a total of 250 g. of sucrose. The day following, a restricted diet *plus* 525 g. of sucrose was taken. The next day, a restricted diet *plus* 150 g. of glucose at breakfast, 150 g. at 10 a.m., 150 g. at lunch and 150 g. at 4.30 p.m. was taken, a total of 600 g. of glucose. The fermentable sugar excretion was 76 mg. and the following day on a restricted diet it was 65 mg. Here by high carbohydrate feeding and stimulation of the pancreas and possibly other carbohydrate-metabolising apparatus on successive days the excretion of fermentable sugar was reduced below the normal on a restricted diet.

Such experiments suggest that the very small quantity of fermentable sugar normally excreted is controlled by the factors operating in general carbohydrate metabolism and that, consequently, the sugar is glucose. Data presented in the third paper substantiate this conclusion. It seems, then, necessary to return to Benedict's view of a continuous "glycuresis" or passage of blood-sugar into the urine. We wish to emphasise, however, that the quantity of sugar passing is so small that it is not detectable by the ordinary qualitative tests and that it is of no clinical importance.

Protein diet preceded by starvation and purgation.

The object of this experiment was to correlate the rate and amount of excretion of fermentable and non-fermentable sugar with that of nitrogen elimination after a protein diet. Greenwald, Gross and McGuire [1927] studied the effect of the ingestion of a protein-fat diet upon the hourly excretion of sugar and nitrogen. They fed mainly eggs as the source of protein and observed no increase in the total sugar output. It should be noted that their total protein intake was relatively small, and also that the present writers have found the non-fermentable sugars from high egg diets quite small. In our experiments preliminary starvation and purgation were employed to remove the residues of previous meals from the intestinal tract. Subject A.C.L. fasted on 8. ii. 29, and took a dose of Epsom salts at 2 p.m. The next day urine samples were collected in 2-hour periods from 5 a.m. to 9 p.m. and 450 g. of lean beef were eaten at 9 a.m. The excretion of non-fermentable and fermentable sugars and of nitrogen for the 2-hour periods was determined. Fig. 1 shows the relations. Non-fermentable sugar was very definitely increased during the 2-hour period following the meat ingestion, while nitrogen remained at about the same level. Both non-fermentable sugar and nitrogen output reached a maximum during the second 2 hours, and fell off sharply 6 hours after the meat and gradually returned towards the basal values. The parallelism of the curves suggests that the non-fermentable sugars arise directly from substances contained in the meat or else are a result of the glandular or other activity concerned with its digestion and absorption. The promptness with which

elimination of non-fermentable sugar begins after meat feeding suggests that some of it may arise endogenously from the organs of digestion. This point has been tested in experiments on the dog from which we conclude that glandular activity does not contribute. These experiments will be given later in the discussion.

A sharp increase in fermentable sugar occurred during the third period after the meat was taken. It is probable also that the glucose of the blood reached a maximum about this time.

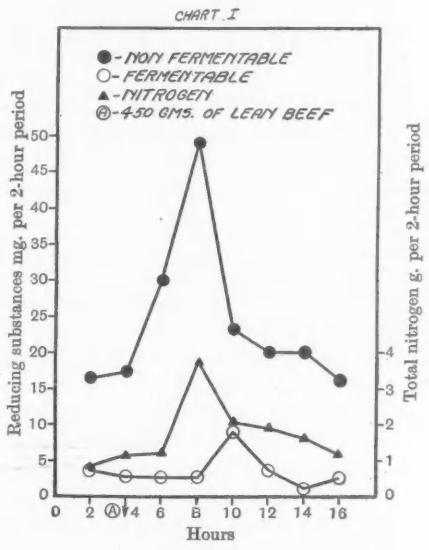


Fig. 1.

Starvation. So far as we are aware there are very few data in the literature on the effect of starvation upon the excretion of non-fermentable and fermentable sugar by human subjects. Harding and Selby [1931] have recently reported the absence of fermentable sugar from normal fasting urines within the limits of the analytical methods employed. We have pointed out in our first paper that the method used by these workers is incapable of estimating with sufficient accuracy the fermentable sugar of starvation urines. We have never failed to find fermentable sugar in the starvation urine of both human beings and the dog, although often in concentrations which the method of Harding and Selby would have failed to detect. We have made observations upon several individuals employing periods of starvation of 1 and 2 days. The results for three subjects are given in Table II. The decrease in non-fermentable sugar was rather less than we had expected and on the second day of starvation this fraction generally showed a slight rise. Fermentable sugar was continually

Table II. *Showing the effect of short periods of starvation upon the excretion of fermentable and non-fermentable sugars of urine.*

Date	Subject	Total N.F. (as glucose) mg.	Total F. (as glucose) mg.	Diet
10. vi. 29	E.S.W.	349	326	Normal
11. vi. 29	„	265	308	Starvation
12. vi. 29	„	274	68	Starvation. True blood-sugar 74 at 5 p.m.
16. vi. 29	H.L.W.	368	142	Normal
17. vi. 29	„	225	38	Starvation
18. vi. 29	„	279	160	Starvation
5. xii. 29	R.H.W.	245	—	High protein
6. xii. 29	„	165	33	Starvation

present. The non-fermentable sugar of starvation must have come from either endogenous sources or food residues remaining in the intestinal tract or both. If food residues were the major factor, a sharp falling off in the output might be expected on the second day of starvation, especially as experiments on the rate of excretion after the ingestion of food show that non-fermentable sugar is eliminated promptly.

Experiments on the dog. In order to establish the effect of prolonged periods of starvation upon the excretion of non-fermentable and fermentable sugar a series of experiments was carried out upon the dog. A female weighing 11·3 kg. and thoroughly house-broken was selected. The animal was kept in a metabolism cage and the urine collected by catheter in 12 or 24-hour periods and preserved in the usual way. No trouble with bladder infection was experienced either on an all-meat diet or during starvation.

The dog was taken from the animal quarters and placed in the cage for several days preliminary to beginning the experiment. Determinations of the daily excretion of non-fermentable and fermentable sugars were then made for a period of 10 days on a diet of 1 lb. of lean horse meat daily in order to establish the normal output. This was followed by 11 days' starvation and then 9 days with a diet of meat as before starvation. Fig. 2 shows the daily excretion of non-fermentable and fermentable sugar throughout the 31 days of the experiment. Nitrogen excretion is also shown for the starvation period and a few days before and after. Several points of interest are brought out by this experiment. In the first place the great variation in non-fermentable sugar on a constant diet in the control period was unexpected, the range being from about 130 mg. to 230 mg. daily, much more than we have ever observed in human subjects on a carefully controlled diet. A chance observation seemed to throw some light upon this anomaly. It was noted that the animal showed much lower excretion of non-fermentable sugar when first taken from the animal quarters and placed in the cage than she did after some days in the cage on practically the same diet. It was also found that there was a rather gradual increase in the excretion upon confinement in the cage for several days with a kind of alternating high and low output but a general trend upward until an

average excretion of about 160 mg. daily was attained. These findings seem to be correlated with the general condition of constipation of the dog. When first taken from the animal quarters where life had been active and bowel movements more frequent and regular, the non-fermentable sugar excretion was low (about 110 mg.). In the cage the dog gradually became constipated so that after a time defaecation occurred only every four or five days, whereupon the non-fermentable sugar excretion increased. More evidence in this direction is to be found in Fig. 2 where it is seen that after the period of starvation was

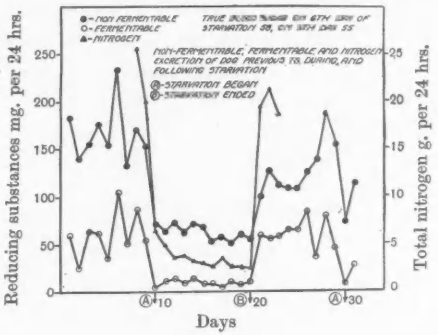


Fig. 2.

terminated, a week was required for the daily excretion of non-fermentable sugar to reach the general level of the pre-starvation period. Presumably this was about the time required to restore the normal movements of the intestinal tract.

It seems justifiable to conclude then that not only do foods contribute directly to the non-fermentable fraction, but that the processes of putrefaction and bacterial action taking place in the intestinal tract determine, in no small degree, the amount and possibly the kind of these substances excreted. These observations seem to represent the first concrete evidence presented in support of the view of Greenwald, Gross and McGuire [1927] that bacterial action in the intestine may contribute to the sugars of urine.

The output of non-fermentable sugar during the period of starvation dropped on the first day to somewhat less than half the previous level and changed very little, on the average, for 11 days. There seems to be a division into two periods, the first of 6 days and the second of 5 during each of which the average excretion was constant but somewhat higher in the first period than in the second. The constancy of the amount throughout starvation seems to suggest an endogenous origin for about a third or half of the non-fermentable sugar normally excreted. It may be that bacterial activity in the intestines produced these substances (or substance) though the constancy of the excretion would seem to preclude this action from playing more than a minor rôle.

During starvation it will be seen that fermentable sugar was continuously present in the urine though in greatly decreased amounts. Immediately after starvation the level rose to a slightly higher average, with less daily variation than before, and after about 6 days the curve resumed the shape of the pre-starvation period. The slightly higher average excretion after starvation may be a reflection of the impairment of carbohydrate metabolism by starvation.

There seems to be some alternation in the daily quantity of both non-fermentable and fermentable sugar excreted, that is, a high value followed by a low. This is not entirely regular and is disrupted for about a week after starvation when it seems to be resumed.

It is of interest to note that hydrolysis of $HgSO_4$-$BaCO_3$ filtrates of dog urine from the seventh day of starvation caused no appreciable change in the non-fermentable sugar and increased the fermentable from 12 to 22 mg. This is in general agreement with results obtained on human subjects and is possibly further evidence for the normal excretion in the urine of non-reducing polysaccharide material.

Glandular stimulation.

Attention has been directed to the promptness with which non-fermentable reducing sugar appears in the urine after taking food. This suggested that some of it may arise from glandular activity of the digestive organs and specifically from the catabolism of nucleic acids. In order to test this hypothesis experiments were made on the fasting dog in which the glands of digestion were stimulated by the sight and smell of food and by the ingestion of pilocarpine. Table III shows clearly that the excretion of both non-fermentable and fermentable sugar remained unchanged. It appears from these data then that the non-fermentable fraction of urine is unrelated to nuclear catabolism, a conclusion reached by Greenwald, Gross and Samet [1924–25] from a study of dogs with turpentine abscesses.

Table III. *Effect of stimulation of the digestive glands of the dog upon the excretion of fermentable and non-fermentable sugars.*

Date	N.F. (as glucose) mg.	F. (as glucose) mg.	Remarks
1. iii. 30	57	11	Starvation
2. iii. 30	54	10	Starvation
3. iii. 30	56	8	Starvation. Stimulated from 9·15–11·15 a.m. by placing hot meat near animal
4. iii. 30	54	10	Starvation. Injected 6 mg. of pilocarpine nitrate intramuscularly at 9 a.m. Severe salivation

Kidney impairment.

A large number of experiments have been carried out on hospital patients afflicted with various pathological conditions. The excretion of non-fermentable sugar for a number of these cases is given in Table IV. It will be noted that

Table IV. *Non-fermentable reducing sugars in the urines of hospital patients.*

No.	Diuretic	24 hour urine cc.	Non-fermentable reducing substances. Total (mg.)	Remarks
1	Salyrgan 1 cc.	1040	161	
		540	81	Patient in hospital with chronic nephritis for 20 weeks
		640	51	
	Salyrgan 0·75 cc.	640	107	Successive days
		540	38	4 days later
	Salyrgan 2 cc.	700	151	
		500	105	Successive days
		—	99	
2		900	81	Cardiac arteriosclerosis
		800	80	
3		810	154	Cardiac decompensation
	Digitalis, 2 days	3900	292	
	Digitalis	1400	266	
4		1310	203	Adult rickets
5		1250	400	Anaemia. P.S.P.* normal
6	Digitalis	2800	420	Aortitis. P.S.P. normal
7		1250	356	Scarlet fever. 8 year child. Casts, otherwise normal
8		830	0	Haemorrhagic nephritis. Non-protein N normal
		600	42	8 days later
9	Digitalis	750	382	Mitral stenosis. No arteriosclerosis. Restricted fluids
		225	166	2 days later
10		850	0	Cardiac nephritis. Non-protein N 56
11	Digitalis	760	167	Cardiac arteriosclerosis
12		1550	163	Post-hypercalcaemia
13		400	188	Diabetes, nephritis. Generalised oedema. P.S.P. 50 %
14		750	382	Determination, 15. iii. 29. Coronary infarct. Non-protein N 48, 13. iii. 29. Non-protein N 100, 18. iii. 29
14		600	324	Determination, 17. iii. 29. Died 18. iii. 29. No gross kidney lesion
Average (14, 9, 5, 6, 7 excluded)			138	
Normal—average of 58 cases			395	

* Phenolsulphonephthalein kidney test.

generally conditions leading to impaired kidney function cause a decreased output of non-fermentable sugar as would be expected. An interesting effect of the drug salyrgan[1] upon the output of non-fermentable sugar by nephritics is shown in that it causes a marked increase. It seems impossible to attribute this action to simple diuresis, because we have found, as did Harding and Van Nostrand [1930], that the output is independent of urine volume.

[1] Salyrgan is the trade name for the diuretic, sodium {*o*-[hydroxymercuric-methoxy-propyl-carbamyl] phenoxy} acetate, manufactured by H. A. Metz Laboratories, Inc., New York.

By way of summary it seems that the physiological factors affecting the excretion of fermentable sugar in normal urine are quite unrelated to those controlling the excretion of the non-fermentable fraction. The fermentable sugar appears to be related to the efficiency of the general carbohydrate metabolism, and probably represents a trivial loss of blood-sugar which escapes from the normal kidney. The non-fermentable fraction, on the other hand, varies with the diet, intestinal stasis and some unknown endogenous process.

Studies relating to the chemical nature of the non-fermentable sugars of urine are planned for the future.

Summary.

A study of factors influencing the excretion of fermentable and non-fermentable sugars in urine by human beings and the dog has been made. The methods of analysis seem to offer more reliable data than have been obtained previously.

Studies on the relation of diet to the elimination of sugars have been made. Non-fermentable sugars apparently are derived in some measure from most common foodstuffs but in particular from dried fruits, apples, honey, *etc.* and carbohydrate-containing foods which have been subjected to high temperatures. This is in agreement with Folin and Berglund's [1922] conclusion that much of the reducing material of normal urine arises from altered carbohydrates of the food. Meat proteins, eggs, cheese, milk, purified carbohydrates, white bread, many fruits and common vegetables contribute somewhat, but not very much, to this fraction.

The excretion of non-fermentable sugar is not increased by high nucleoprotein diets or by the ingestion of yeast nucleic acid. Stimulation of the glands of digestion does not increase the excretion of non-fermentable sugar. Nucleoprotein metabolism seems to be entirely unrelated to this material.

In starvation experiments on both man and the dog, the excretion of non-fermentable sugar continued but at a somewhat lowered level. In the dog one-third to one-half may be endogenous and in man possibly more.

From experiments on the dog it is shown that intestinal stasis causes an increased output of non-fermentable sugar and it is concluded that bacterial action and putrefaction in the intestine may be considerable factors.

The non-fermentable sugar of urine is very little changed by hydrolysis.

Hospital patients with kidney impairment show a decreased output of non-fermentable sugar in the urine.

Fermentable sugar was found in all normal urines and in decreased amounts in the urines of persons during starvation. It was present in dog urine throughout a prolonged period of starvation.

The excretion of fermentable sugar in normal urine seems to be related to the condition of activity of the pancreas and of the general carbohydrate

metabolism. Conditions which lower the carbohydrate tolerance increase the fermentable sugar excreted and *vice versa*. This suggests that the small quantity of fermentable sugar of normal urine is glucose and that Benedict's idea of a continual passage of blood-sugar into the urine is correct. The amount present is undetectable by ordinary qualitative tests and of no clinical importance.

Evidence is presented that normally there is excreted in urine non-reducing polysaccharide material which upon hydrolysis yields fermentable sugar.

The writers are indebted to Mr George H. Curtis and Mr Arthur Brandon for aid in carrying out some of the experiments, and to Prof. Shaffer for helpful advice and criticism.

REFERENCES.

Benedict and Osterberg (1918). *J. Biol. Chem.* **34**, 209.
—— —— (1921). *J. Biol. Chem.* **48**, 51.
—— —— and Neuwirth (1918). *J. Biol. Chem.* **34**, 217.
Dann and Chambers (1930). *J. Biol. Chem.* **89**, 675.
Eagle (1926–27). *J. Biol. Chem.* **71**, 481.
Folin and Berglund (1922). *J. Biol. Chem.* **51**, 213.
—— and Wu (1920). *J. Biol. Chem.* **41**, 367.
Greenwald, Gross and McGuire (1927). *J. Biol. Chem.* **75**, 491.
—— —— and Samet (1924–25). *J. Biol. Chem.* **62**, 401.
Harding and Van Nostrand (1930). *J. Biol. Chem.* **85**, 765.
—— and Selby (1931). *Biochem. J.* **25**, 1815.
Heinbecker (1928). *J. Biol. Chem.* **80**, 461.
Höst (1923). *J. Metabol. Res.* **4**, 315.
Patterson (1926). *Biochem. J.* **20**, 651.
Peterson and West (1929). *Amer. J. Physiol.* **90**, 470.
Scharles and West (1931). *J. Biol. Chem.* **93**, 359.
Shaffer and Hartmann (1920–21). *J. Biol. Chem.* **45**, 365.
Somogyi (1927). *J. Biol. Chem.* **75**, 33.
Van Slyke and Hawkins (1929). *J. Biol. Chem.* **83**, 51.
West and Peterson (1932). *Biochem. J.* **26**, 1720.

CCV. THE SUGARS OF URINE.

III. THE CHEMICAL NATURE OF THE FERMENTABLE SUGAR OF NORMAL AND STARVATION URINE.

By EDWARD STAUNTON WEST and ALEXANDER STEINER.

From the Laboratory of Biological Chemistry, Washington University School of Medicine, Saint Louis, Missouri.

(*Received August 2nd, 1932.*)

That a part of the sugar of normal urine is fermentable or rather yeast-utilisable seems to have been established by the work of Van Slyke and Hawkins [1929], Peterson and West [1929], West and Peterson [1932], and West, Lange and Peterson [1932]. These last workers have also uniformly found it in starvation urines. Harding and Selby [1931] observed fermentable sugar in urines after carbohydrate and fruit diets but not in starvation urines. The failure of Harding and Selby to find fermentable sugar in starvation urines has been discussed in the second paper of this series and shown to be due probably to their analytical procedure.

The methods of analysis used by all the above workers indicate only that a part of the reducing power of urine is lost as a result of yeast action. They do not prove that a sugar, fermentable in the sense of yielding carbon dioxide and alcohol, is present. In fact, Lund and Wulf [1926] failed to observe any carbon dioxide production from normal urine when treated with pure yeast in a Barcroft manometer. These workers conclude that glucose is not a constituent of normal urine. Various investigators have prepared osazones of the urine-sugars in attempts to prove or disprove the presence of glucose. Of these Geelmuyden [1915], Höst [1923] and Patterson [1926] have reported failures to isolate *d*-phenylglucosazone from normal urines. Hassan [1928] reported the preparation of typical glucosazone crystals from many, but not all, normal urines. Greenwald, Gross and McGuire [1927] have called attention to the possibility of forming glucosazone from non-fermentable sugar derivatives of urine, especially the anhydro-sugars. Everett and Sheppard [1932] have pointed out some of the sources of error in attempts to identify glucosazone crystals among the osazones formed from urine-sugars.

It should be emphasised that neither the production of carbon dioxide under the action of yeast nor the isolation of glucosazone establishes the presence of glucose in urine. Glucose, fructose, mannose and some of the

anhydro-sugars give rise to glucosazone, and glucose, fructose, mannose and some disaccharides are fermented by yeast.

The failure of Lund and Wulf to obtain carbon dioxide production in their fermentation of normal urine may be related to two factors. They used 1 cc. of undiluted, untreated urine and added 0·5 cc. of a 1 % phosphoric acid solution to it before fermentation to adjust the p_H. Undiluted, untreated urine contains a large amount of non-sugar material which may inhibit yeast action on small quantities of sugar. Also their adjustment of p_H was uncertain, doubtless varying from sample to sample as the buffer content of the urines varied. They make no statements as to the p_H of any of their samples. Some normal urines may contain only 5–8 mg. of glucose per 100 cc. and in such cases they were working with 0·05–0·08 mg. of sugar. This quantity would produce from 11 to 17 mm.^{3} of carbon dioxide which may have been absorbed by the buffers of their solutions without registering on the manometer.

Because of the unsatisfactory state of the problem, resulting from inadequate methods, the writers have tried a different approach in an attempt to establish the presence or absence of glucose in both normal and starvation urines. It is a well-established fact that sugars are fermented at different rates and it is possible, by use of the Warburg apparatus, to obtain a characteristic curve of carbon dioxide production for each fermentable sugar. In order that such a procedure may be successfully applied to urine it is advisable that the urine be treated to remove most of the nitrogenous and other non-sugar constituents and that the p_H of the solution be accurately adjusted. Also the solution fermented should contain much more sugar than undiluted, normal urine. These demands have been met in the following way. $HgSO_4$-$BaCO_3$ filtrates of large quantities of urine (800 cc.) were prepared according to the procedure already outlined [West and Peterson, 1932]. These filtrates were evaporated to dryness, taken up in a small quantity of H_2O and precipitated with alcohol to remove salts. The alcoholic solutions were evaporated to dryness, taken up in H_2O and again precipitated with $HgSO_4$-$BaCO_3$. Evaporation of the filtrates and precipitation with alcohol were repeated and the alcoholic solution, free of salts, was evaporated to dryness. The residue was then taken up in 0·15 M NaH_2PO_4. By this procedure a sugar solution of the desired sugar concentration and p_H and relatively free from nitrogenous and other non-sugar substances is obtained. Suitable quantities of these solutions were fermented with washed yeast in the Warburg apparatus and the rate of carbon dioxide formation observed. Urine-sugar residues were also taken up in 0·5 N H_2SO_4 and hydrolysed before running fermentation curves. Solutions of glucose, fructose and mannose of the same reduction equivalents as the fermentable sugar of the urine samples were fermented in exactly the same manner simultaneously. In this way a series of curves of the fermentable hexoses was obtained under the same conditions of concentration (reduction equivalent), p_H and temperature as for the fermentable sugar of the urine samples.

Experimental.

Urine samples.

The daily fermentable sugar excretion of the three subjects who supplied urine for the following work was normal, there being no indication of a low renal threshold. For the collection of normal urine samples the subject was placed on a sugar- and fruit-free diet for 2 days, the sample being taken the second day. This was done to eliminate the possibility of sugar appearing in the urine from the ingestion of fruit juices *etc.* as found by Harding and Selby [1931]. For starvation urines the subject ate supper and began collecting the sample the next day about 15 hours after the last meal, which was sugar- and fruit-free. Urines were preserved during collection and for the short period before use by means of toluene.

Preparation of urine-sugars for fermentation.

Two portions of 400 cc. of urine are precipitated as follows and the filtrates combined.

400 cc. of urine and 1800 cc. of H_2O are placed in a 6-litre Florence flask. To this are added 600 cc. of a saturated solution of $HgSO_4$ in 10 % H_2SO_4 (about 28 g. of $HgSO_4$ per 100 cc.) with rapid mixing. Solid precipitated $BaCO_3$ is then quickly added and the solution neutralised as rapidly as possible by continual shaking and addition of further portions of $BaCO_3$. The reaction is complete when CO_2 ceases to be evolved and the solution no longer reddens blue litmus. The precipitate is filtered off on washed papers with suction, and the filtrate (4000–5000 cc.) is treated with 2 cc. of conc. H_2SO_4 per litre. Mercury is removed from the filtrate by H_2S and the latter is blown off with air. The solution is then neutralised with pure $BaCO_3$ and filtered at the pump through washed papers. A few drops of conc. H_2SO_4 are added to the filtrate and a little NaH_2PO_4 so that it reacts acid to litmus and alkaline to Congo red. A little toluene is added to the solution which is then evaporated to dryness at a temperature of 30–35° under reduced pressure, with a stream of CO_2 passing through it. At intervals the evaporation is interrupted and more NaH_2PO_4 added, if necessary, to maintain a faintly acid reaction. The residue is taken up in about 50 cc. of H_2O, precipitated with 1000 cc. of alcohol, and after standing overnight the solution is filtered and the alcohol evaporated *in vacuo* at 30–35°. The residue is taken up in 165 cc. of H_2O and precipitated with 45 cc. of the $HgSO_4$ reagent, neutralised with $BaCO_3$ and excess mercury removed with H_2S in the usual way. The filtrate is neutralised with $BaCO_3$ and the reaction made faintly acid with a drop or two of conc. H_2SO_4 and a little NaH_2PO_4 and the solution evaporated to dryness as in the first case. The residue is taken up in 25 cc. of H_2O and precipitated with 500 cc. of alcohol. After standing overnight the solution is filtered and divided into two equal parts. Both are evaporated to dryness *in vacuo* at 30–35°. One residue is taken up in 25 cc. of 0·5 *N* H_2SO_4 and hydrolysed in a boiling water-bath for 5 hours. The solution

is then diluted to 300 cc. with H_2O, neutralised with pure $BaCO_3$, filtered through washed papers at the pump, the precipitate washed with 50 cc. of H_2O and the washings combined with the filtrate. The solution is then rendered faintly acid with NaH_2PO_4 and evaporated to dryness *in vacuo* in the usual way. The residue is taken up in about 20 cc. of H_2O, precipitated with 300 cc. of alcohol, filtered, and the alcoholic solution evaporated *in vacuo* as above. The hydrolysed residue remaining is taken up in 15–20 cc. of 0·15 *M* NaH_2PO_4 as is also the other residue which has not been hydrolysed. Fermentable sugar is then determined in the solutions, after proper dilution, by the method of West and Peterson [1932]. This generally amounts to 0·5 mg. or more per cc. of solution.

In order to determine whether the fermentable sugar might arise from the filter-paper used, a water blank was run through in the same way as urine. No fermentable sugar was found either before or after hydrolysis.

The $HgSO_4$-$BaCO_3$ filtrates are kept slightly acid during evaporation, otherwise the alkalinity produced as the solutions concentrate may bring about interconversion of related sugars.

There is considerable loss of sugar in the process, probably referable to retention in the precipitates and possibly to esterification with the phosphate added during evaporation. When only one $HgSO_4$ precipitation is performed and evaporation is done without phosphate, the loss is small.

The nitrogen content of such solutions generally amounts to 0·5 mg. or less per cc. or about 10 mg. total N in the entire solution as contrasted with about 4000 mg. in the original untreated urine. It has been found desirable to use the double precipitation with $HgSO_4$-$BaCO_3$ in order more efficiently to remove non-sugar materials which interfere with and inhibit fermentation.

Fermentation in the Warburg apparatus.

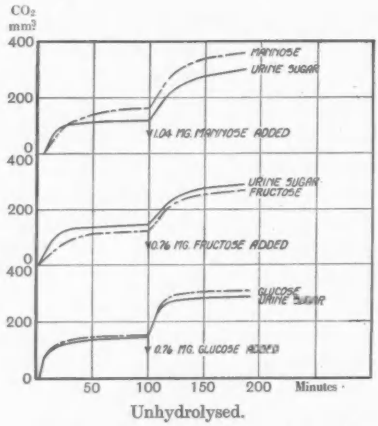
The fermentations were done in the usual way. Vessels with two side-arms were used. Three series were set up in which the urine-sugars were fermented simultaneously with glucose, fructose and mannose, all made up in 0·15 *M* NaH_2PO_4 solution. These control sugar solutions were prepared so that there was present in 0·2 cc. a quantity of sugar equivalent in reduction value to the fermentable urine-sugar. The arrangements were as follows. 1–2 cc. of the urine-sugar solution were placed in the vessel. Sufficient 0·15 *M* NaH_2PO_4 was then added to bring the volume to 3 cc. 0·2 cc. of a 20 % suspension of washed Fleischman's yeast was placed in one side-arm, and 0·2 cc. of the control sugar (glucose, fructose or mannose) in the other. The controls of glucose, fructose and mannose were prepared in the following way. 0·2 cc. of the sugar solution was placed in the vessel with 2·8 cc. of NaH_2PO_4 solution. 0·2 cc. of the sugar solution was also placed in one side-arm, and 0·2 cc. of the yeast suspension in the other. The vessels, with manometers, were then equilibrated with nitrogen and placed in the constant temperature bath at 30°. A thermobarometer containing 3·4 cc. of 0·15 *M* NaH_2PO_4 solution was placed in the bath simultaneously. After temperature equilibrium was reached the yeast

was tipped into the vessels and readings taken at 5 minute intervals throughout the period of rapid fermentation and at 10 minute intervals thereafter. Upon completion of the first fermentation the sugar solutions in the side-arm were tipped into the vessels and readings taken as before.

A fermentation was performed in which one side-arm contained saturated $Ba(OH)_2$. A large quantity of $BaCO_3$ separated indicating that the gas produced from the fermentable urine sugar was carbon dioxide.

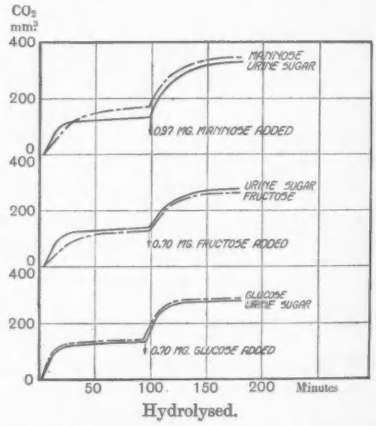
Discussion.

Figs. 1–8 summarise the results obtained on two normal urines from different subjects and similarly two starvation urines. The curves are plotted in pairs showing simultaneous fermentation of urine-sugar and an equivalent concentration (from reduction value) of glucose, fructose and mannose. The second parts of the curves represent the fermentations of sugar (equivalent to that in the original urine and control sugar solutions) added to the previously fer-



Unhydrolysed.

Fig. 1. Normal urine—I. Sugar- and fruit-free diet. Reducing power of sample = 0·758 mg. glucose.



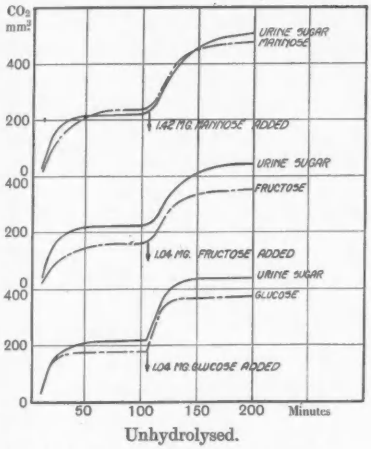
Hydrolysed.

Fig. 2. Normal urine—I. Sugar- and fruit-free diet. Reducing power of sample = 0·70 mg. glucose.

mented urine and control sugar solutions. This was done to determine whether there were non-fermentable substances in the urine samples which interfered with the rate of fermentation. It will be seen that the sugar added to the previously fermented urine-sugar solution fermented essentially similarly to that added to the fermented control.

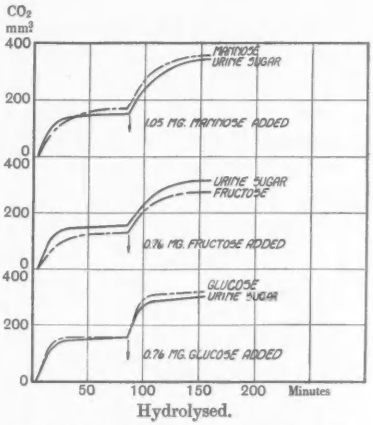
In all cases the curves of fermentation of the urine-sugar follow closely those of an equivalent quantity of glucose throughout the period of rapid fermentation, and in three cases of normal urine they run closely parallel throughout. On the other hand, the fermentation curves of fructose and mannose show entirely different rates of carbon dioxide production in all cases.

Experiments on maltose were also carried out and it was found to ferment much more slowly than the urine-sugar. We interpret these findings as evidence that



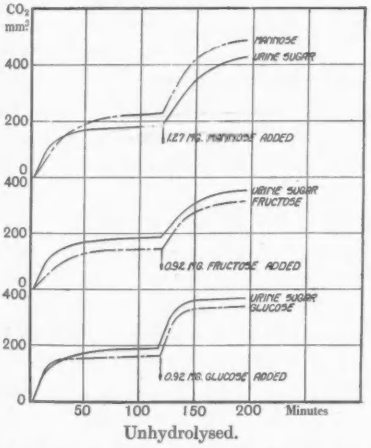
Unhydrolysed.

Fig. 3. Starvation urine—I. Reducing power of sample = 1·04 mg. glucose.



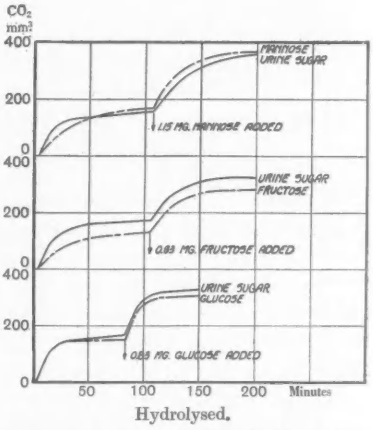
Hydrolysed.

Fig. 4. Starvation urine—I. Reducing power of sample = 0·76 mg. glucose.



Unhydrolysed.

Fig. 5. Normal urine—II. Sugar- and fruit-free diet. Reducing power of sample = 0·92 mg. glucose.



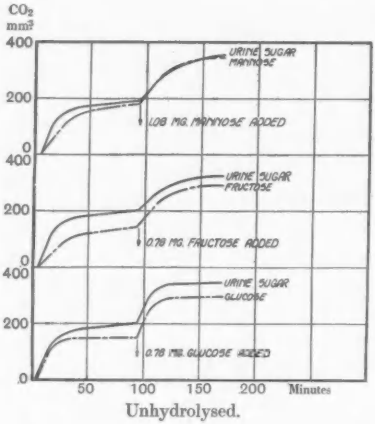
Hydrolysed.

Fig. 6. Normal urine—II. Sugar- and fruit-free diet. Reducing power of sample = 0·83 mg. glucose.

both normal and starvation urines contain fermentable sugar in the strict sense of the term and that this sugar is glucose.

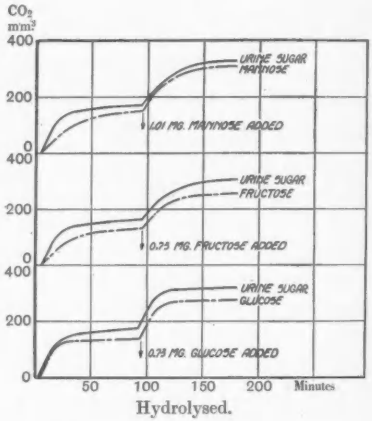
The divergence found in the final stages of fermentation in some cases may be referable to several factors. Generally the urine-sugar curve rises higher and

with a more gradual slope than the glucose curve. This may possibly be due to slow decarboxylation of an organic acid, or the slow fermentation of a disaccharide or to the stimulating effect of certain non-sugar substances upon the yeast. There may also have been slight errors in determining the fermentable sugar of the urine-sugar solutions. The slopes of the curves, however, do not indicate that this is an important factor. We have found that the addition of a little $(NH_4)_2SO_4$ or pyruvate to a glucose solution gives curves similar in form to these elevated urine-sugar curves, and are inclined to consider them, in part at least, due to the effect of such or similarly acting substances.



Unhydrolysed.

Fig. 7. Starvation urine—II. Reducing power of sample = 0·78 mg. glucose.



Hydrolysed.

Fig. 8. Starvation urine—II. Reducing power of sample = 0·73 mg. glucose.

It should be pointed out that pyruvate is not decarboxylated by yeast under the conditions of our experiments but if mixed with glucose slightly more CO_2 is evolved than from the glucose alone. The effect was removed in two cases by hydrolysis and not in another. From this it seems unlikely that it can be referred to the presence of a slowly fermentable disaccharide. Sugars of this nature would not be expected in the urines we used unless of an endogenous origin.

The glucose in urine found by us probably did not arise from the hydrolysis of anhydro-sugars referred to by Greenwald, Gross and McGuire [1927]. These should not exist in either normal urines from a sugar- and fruit-free diet, or starvation urines.

Summary.

Evidence is presented that both normal and starvation human urines contain fermentable sugar in the strict sense of the term and that this sugar is glucose. This evidence is based upon the fermentation rates of purified urine-

sugars in the Warburg apparatus simultaneously with glucose, fructose and mannose controls. The rate of carbon dioxide production from the urine-sugars parallels that from glucose but not those from mannose or fructose.

The writers are indebted to Mr R. C. Norris for technical assistance.

REFERENCES.

Everett and Sheppard (1932). *J. Biol. Chem.* **96**, 431.
Geelmuyden (1915). *Morsk. Mag. Laegevidenskaben*, **13**, 985.
Greenwald, Gross and McGuire (1927). *J. Biol. Chem.* **75**, 491.
Harding and Selby (1931). *Biochem. J.* **25**, 1815.
Hassan (1928). *Biochem. J.* **22**, 1332.
Höst (1923). *J. Metabol. Res.* **4**, 315.
Lund and Wulf (1926). *Biochem. J.* **19**, 538.
Patterson (1926). *Biochem. J.* **20**, 651.
Peterson and West (1929). *Amer. J. Physiol.* **90**, 470.
Van Slyke and Hawkins (1929). *J. Biol. Chem.* **83**, 51.
West and Peterson (1932). *Biochem. J.* **26**, 1720.
—— Lange and Peterson (1932). *Biochem. J.* **26**, 1728.

CCVI. THE IMMUNOLOGICAL REACTION TO THE ENZYME PAPAIN.

By ROBERT PETRIE WALTON and CORA MAE SEGURA.

Department of Pharmacology, School of Medicine, Tulane University and Department of Chemistry, Newcomb College, New Orleans.

(*Received August 9th, 1932.*)

Reports have been highly contradictory with respect to the possible development of increased anti-enzymic activity in the serum of a subject which has received repeated injections of a given enzyme preparation. Wells [1925], in reviewing the subject, considers the general thesis very much open to doubt. Probably the most definite evidence of a positive nature is the work by Kirk and Sumner [1931; 1932] with crystallised urease preparations. This particular enzyme is much more stable in the presence of serum than most enzymes and such a property would seem to be a requisite for the production of an anti-enzyme. Northrop [1930], for instance, failing to obtain anti-enzymic effects with a crystallised pepsin preparation, attributed the failure to the recognised instability of pepsin in blood-serum. The vegetable enzyme, papain, by virtue of its relative stability in serum [Walton, 1931, 1, 2] is of special interest in this regard. In the previous studies [Ehrenreich, 1900; Stenitzer, 1908; Achalme, 1901; Pozerski, 1909] concerning the possible development of an antipapain effect proteolytic assay methods have been used which were evidently of slight sensitiveness. For instance, the presence of fluctuating amounts of serum-proteases in the same animal was not recognised in such studies. Ehrenreich [1900] and Achalme [1901] were unable to obtain antipapain effects with rabbits and guinea-pigs, while Stenitzer [1908] reported an anti-enzymic effect in one case with a goat. On the basis of these considerations, the problem was reinvestigated, as reported here, using a method of proteolytic assay which is unusually accurate at relatively high dilutions. The sensitiveness of this particular method was such that the development of a relatively slight anti-papain effect should have manifested itself in the recorded measurements.

Procedure.

Two healthy dogs of 8 and 10 kg. weight were each subjected to 20 intravenous and 4 intraperitoneal injections of a papain preparation during a period of 97 days. Venous blood was withdrawn at intervals and the serum used in an assay of its effect on the proteolytic activity of a solution of the papain. Samples were always taken before any injections given the same day. Intravenous

injections were borne without apparent immediate effects, while intraperitoneal injections caused considerable discomfort for a few minutes. During the course of the injections the animals lost weight and developed hemorrhagic skin manifestations, but maintained voracious appetites. On discontinuance of the injections they returned to normal.

Sterile papain for injection was obtained by pressure filtration of glycerol-water solutions through Berkefeld candles and preservation of the filtrates in sealed ampoules. Fresh filtrates, prepared about every 3 weeks, were sufficiently stable, as judged by measurements in this work and by a previous standardisation study of the same procedure [Walton, 1931, 1, 2; 1932]. The stock preparations of Parke Davis papain, whose activity has been defined in this same study, were used throughout. Injections of 1 cc. of filtrate (activity equal to about 40 mg. dry powder) are indicated in Fig. 1 by a single arrow and injections of 2 and 3 cc. by double and triple arrows, respectively.

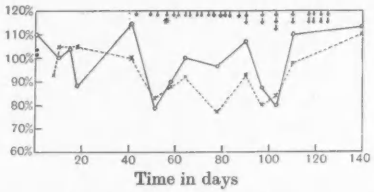


Fig. 1. Ordinates: percentage original activity of a 1–1000 papain solution after being mixed with serum (10 %).

↓ Injection of a papain solution equal in activity to 40 mg. dry powder.
● Observations on normal dogs.

The determinations of proteolytic activity were carried out essentially according to the photographic film method as first described by Gates [1930] and by Gilman and Cowgill [1930]. The technique previously detailed by one of us [Walton, 1931, 1, 2; 1932] was modified in that no fixed temperature was used, but rather any constant temperature in the neighbourhood of 30° which would carry the standard digestion solutions (1–1000 papain) to the end-point (50 % digestion of the gelatin film) in about 20 minutes. Standards and unknowns were always run together at the same temperature. The series of digestion mixtures were sufficient in number to ensure an accuracy of $\pm$ 5 %. Papain at a 1–1000 concentration in a solution of 1 % sodium citrate was compared in its digestive activity with solutions of papain at similar concentrations but containing 10 % serum. If the serum was completely inert in this system, its 10 % mixture with a standard solution would result in a solution exhibiting 90 % of the activity of the standard solution. Observations with normal serum demonstrate the frequent presence of proteases in sufficient strength to manifest activities as high as 115 % of the standard in mixtures of this sort.

Possible serum changes due to varying intervals between time of sample taking and time of assay were shown to be without significance. Samples of

the two sera assayed at 1½ and 5 hour intervals after withdrawal showed no variation outside the accepted experimental error. These and all samples used in the study were kept on ice during this interval. A more drastic test of this point was made by heating the two sera for 1 hour at 60° before mixing with papain. Whereas the mixtures of papain with unheated sera exhibited activities of 100 % and 110 %, the mixtures with the heated sera exhibited activities of 85 % and 100 %, respectively.

Varying intervals between the time at which the serum was mixed with papain and the time of assaying the mixture were likewise shown to be without significance. Samples of the two sera mixed with papain and assayed immediately manifested substantially the same effect when assayed after the mixture had stood for 1 hour at 25°.

One comparison of the two sera taken before and after feeding showed a decrease in the proteolytic activities of the mixtures after feeding. Sera obtained before feeding exhibited activities of 85 % and 110 % in the usual mixtures with papain, while those obtained after feeding exhibited activities of 77 % and 95 %, respectively. During the course of the study, samples were taken at a regular period about 4 hours after feeding, and, as these figures indicate, some such routine procedure is essential.

Results.

Results are illustrated in Fig. 1. The normal fluctuations of serum-proteases are evidently very appreciable and any study of this type must account for such, either by subtracting the protease effect of the serum at each determination or by obtaining a sufficient number of normal determinations to establish an average. It is apparent from the general direction of the curves that the development of an antipapain effect, if present at all, is very transitory and of slight magnitude. The occasional drop in the curves may as well be due to a disturbed physiology as to an immunological reaction.

A violent reaction which occurred in both dogs on the fourth injection was of an anaphylactic nature in every observable characteristic except for the unusually brief incubation time. No further reactions of this sort occurred, although some of the subsequent injections were 3 times the dosage of that which provoked the reaction.

The sera gave precipitin reactions with filtered solutions of the papain. However, observations of this sort are complicated by the fact that normal sera gave apparent precipitin reactions with papain solutions.

Towards the latter part of the study, serum-papain mixtures of the proportions previously described were assayed before and after centrifuging off the precipitate obtained by their mixture. After removing the precipitates, which were of very appreciable bulk, the supernatant fluids exhibited substantially the same proteolytic activity as the uncentrifuged portions of the same mixture. Apparently, the proteolytic component of the papain preparations is not subject to such precipitation.

SUMMARY.

A series of observations has demonstrated the absence of any consistent tendency toward development of an anti-enzymic agent on continued injection of papain. Factors influencing the determinations of such possible effects are evaluated.

REFERENCES.

Achalme (1901). *Ann. Inst. Pasteur*, **15**, 737.
Ehrenreich (1900). Inaugural Dissertation, Würzburg.
Gates (1930). *Science*, **72**, 147.
Gilman and Cowgill (1930). *J. Biol. Chem.* **88**, 743.
Kirk and Sumner (1931). *J. Biol. Chem.* **94**, 21.
—— (1932). *Proc. Soc. Exp. Biol. Med.* **29**, 712.
Northrup (1930). *J. Gen. Physiol.* **13**, 739.
Pozerski (1909). *Ann. Inst. Pasteur*, **23**, 205, 321.
Stenitzer (1908). *Biochem. Z.* **9**, 383.
Walton (1931, 1). *J. Pharm. Exp. Ther.* **43**, 487.
—— (1931, 2). *Proc. Soc. Exp. Biol. Med.* **28**, 922.
—— (1932). *J. Lab. Clin. Med.* **17**, 459.
Wells (1925). Chemical pathology, 5th ed.

CCVII. THE EFFECT ON SOLUTIONS OF DIPHTHERIA TOXIN AND DIPHTHERIA ANTITOXIN OF CONTACT WITH CERTAIN SURFACES.

By PETER JOSEPH MOLONEY and EDITH MARGERY TAYLOR.

From the Connaught Laboratories, University of Toronto.

(*Received August 13th, 1932.*)

In some preliminary experiments on the stability of diphtheria toxin we had observed that when a dilute solution of diphtheria toxin is allowed to stand in a cylindrical glass vessel, the rate of disappearance of toxicity is greater the smaller the volume of the solution. This phenomenon probably depends, in part at least, on the greater ratio of air surface to volume of the smaller volume of liquid[1], and may depend in part on the greater ratio of glass surface to volume.

The original purpose of the work reported here was the investigation of the effect of glass surfaces on diphtheria toxin.

In order to obtain a relatively large ratio of glass surface to volume of liquid, the following arrangement was used. Pairs of pyrex glass test-tubes were selected such that for each pair one tube fitted rather snugly into the other. The outer tubes were approximately 15 cm. in length by 1·8 cm. in diameter, and the inner tubes 13 cm. in length by 1·5 cm. in diameter; the volume of liquid contained between the two glass surfaces was not greater than 2 cc. With such fitted tubes it was possible to have a ratio of surface to volume in the outer compartment at least 20 times as great as in the inner tube.

Before the tubes were used they were allowed to stand in hot sodium chromate-sulphuric acid solution for at least 2 hours, then rinsed several times in tap-water and finally in distilled water.

To carry out a test with such tubes, the inner tube of a pair was removed, 10 cc. of toxin measured in a pipette and about 2·5 cc. expelled into the outer tube. The inner tube was then replaced and pushed to the bottom, thus expelling excess liquid into the inner tube. The remainder of the toxin in the pipette was then run in. The tubes containing the toxin were kept at room temperature for about 16 hours, after which the contents of the inner and outer compart-

[1] Glenny *et al.* [1925] have shown that dilute solutions of diphtheria toxin containing phenol lose in toxicity when shaken; and that this loss of toxicity can be ascribed to the detoxifying action of phenol, the concentration of which is markedly increased in the foam [Pope, 1927]. The toxin solutions used in the experiments recorded in this paper contained no added preservative. Such solutions are also unstable to shaking.

ments were tested for toxicity by intradermal injection into normal guinea-pigs.

In these experiments it was desirable, on account of the possibility of bacterial contamination, to use aseptic technique. To this end the dry fitted tubes were plugged with cotton-wool (non-absorbent) and sterilised by heating in an air-oven at 175–185° for 1 hour. When dilute diphtheria toxin solution was put in the inner and outer compartments of the sterilised tubes, and kept at room temperature for 16 hours, it was found that the toxicity of the solutions in the outer compartments had decreased markedly as compared with those in the inner compartments. When, however, this experiment was carried out using tubes which during sterilisation had been covered with aluminium caps in place of cotton plugs, no such marked difference in the toxicity of the contents of the two compartments was observed.

A series of experiments was then carried out to determine the effect of a variety of treatments on the tubes prior to the addition of the toxin. Tubes were heated in the air-oven at 175°–185° for 1 hour—some uncovered, some covered with aluminium caps, some plugged with cotton-wool (non-absorbent), some with a small piece of cotton-wool at the bottom of the outer and the inner tubes in addition to cotton plugs, some plugged with filter-paper and some with a piece of filter-paper in the bottom of the outer and inner tubes in addition to the paper plugs. Other tubes were coated with a film of paraffin wax.

The diluted toxin which was put into these tubes was made by diluting a sterile toxic broth filtrate (80,000 M.R.D. per 0·1 cc.) with buffered saline at p_H 7·02 (1–4 dilution of McIlvaine's phosphate buffer, sodium chloride added to a concentration of 0·85 %).

Table I. *Disappearance of toxin in fitted tubes.*

Treatment of tubes prior to addition of toxin	1/4000 diln. toxin No. 461		1/2000 diln. toxin No. 461	
	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume
Heating with no covering	—	—	19, 19, 19, 21, 17, 20	19, 20, 24, 21, 20, 21
Heated with metal caps	15, 16	19, 20	21, 21, 21	21, 21, 19
Heated with cotton plug in top and cotton at bottom of both compartments	0, 0	19, 18	0, 0, 0, 0, 0, 0, 0, 0	20, 25, 20, 20, 20, 24, 21, 22
Heated with cotton plug in top only	0, 0	20, 19	14, 12, 13F, 11, 0	24, 23, 23, 25, 23
Heated with ash-free filter-paper over top and at bottom of both compartments	—	—	0, 0, 0, 0	20, 23, 21, 24
Heated with ash-free filter-paper over top only	—	—	9F, 15, 11F	23, 23, 21
Tubes coated inside and out with washed paraffin wax	0, 0, 0	16, 16, 18	0, 0, 0, 0 0, 0, 0, 0	19, 19, 20, 20 22, 21, 24, 20

Note. The numbers in all the tables represent the average diameter in mm. of intradermal reactions on guinea-pigs 48 hours after injection. The experiments were set up at least in duplicate; each number represents a test on material from an individual compartment.

F=faint reaction.

After the diluted toxin had been added to the tubes, these were kept for 16 hours at room temperature. The contents were then tested by injecting 0·1 cc. amounts of the solutions intradermally into guinea-pigs. The results are summarised in Table I.

It will be observed that there is evidence of detoxification only in the case of toxin solutions from the outer compartments—large surface to volume; and further, that marked detoxification has taken place only in the tubes coated with paraffin wax and in the tubes heated with cotton and with paper. The results with the cotton and with the paper indicate that during sterilisation some volatile substance from cotton-wool or filter-paper is deposited on the glass and that this new surface is much more effective in detoxifying diphtheria toxin than is clean glass.

The conditions under which glass tubes plugged with cotton-wool are heated, prior to the addition of toxin solution, affects the capacity of the glass surface for bringing about the disappearance of toxin. This is shown in the results given in Table II. Before the tubes were heated under the various conditions indicated, a piece of cotton-wool was put into the inner and outer compartments of each pair of tubes, in addition to a cotton plug in the top. The cotton was removed from the inner and outer compartments before toxin solution was added.

Table II. *Disappearance of toxin in fitted tubes previously heated with cotton-wool at various temperatures.*

		1/1000 diln. toxin No. 461		1/2000 diln. toxin No. 461	
		Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume
Temperature	Time of heating				
78°–80° hot air	1½ hrs.	20, 17, 0	23, 22, 24	15, 16, 0	22, 21, 23
132°–133° hot air	1½ hrs.	0, 15, 11	21, 20, 20	0, 0, 0	20, 18, 20
165°–185° hot air	1½ hrs.	0, 0, 0	20, 22, 21	0, 0, 0	22, 18, 20
		0, 0, 0	24, 27, 20		
195°–218° hot air	1½ hrs.	0, 12, 0	21, 19, 20		
Autoclaved at 16 lbs. pressure	30 mins.	14, 0, 16	20, 20, 20	0, 0, 0	17, 20, 20

The maximum disappearance of toxin was obtained in tubes which had been heated at 165°–185°.

That cotton-wool itself is not effective in bringing about the disappearance of toxin is shown in Table III.

Table III. *Effect of cotton-wool on diluted diphtheria toxin.*

	Size of reaction
Unheated cotton	19
Cotton heated at 175°–185° for 1½ hrs.	17
Cotton heated at 200°–218° for 1½ hrs.	18
Cotton heated in autoclave at 16 lbs. pressure for 30 mins.	19
Toxin solution control (not in contact with cotton-wool)	19

The cotton-wool used, both heated and unheated, was placed in test-tubes and just sufficient diluted toxin added to cover it. When the tubes had stood overnight at room temperature the toxin solutions were tested by intradermal injection into guinea-pigs.

The detoxifying capacity of glass is markedly increased after contact with light petroleum[1]. This is shown in Table IV. The following experimental details concern this Table. Clean fitted tubes, covered with metal caps, were heated for 1 hour at 175–185°. A number of organic liquids—light petroleum, ethyl

Table IV. *Effect of certain organic liquids on the detoxifying capacity of glass.*

	1/2000 dilution toxin No. 461	
	Large surface / volume	Small surface / volume
Clean tubes covered with metal caps and heated in hot air-oven at 180°	24, 20, 21	20, 19, 22
Tubes treated with light petroleum and rinsed	0, 0, 0, 0, 0, 0, 0, 0, 0	20, 20, 20, 21, 25, 20, 19, 20, 18
„ ethyl ether „	18, 16	20, 18
„ toluene „	17, 15, 19	21, 20, 18
„ xylene „	17, 15, 13	17, 21, 22
„ benzene „	18, 13, 16	24, 25, 22
„ ethyl alcohol „	0, 16	21, 24

ether, *etc.*—were then put into the inner and outer compartments of the fitted tubes, the tubes stoppered with corks and left for 16 hours at room temperature. The organic liquids were then removed and the tubes rinsed 15 times with water. After this a dilute solution of toxin was put in the inner and outer compartment of each pair and the tubes left at room temperature for a further period of 16 hours. The contents were then tested for toxicity by intradermal injection into guinea-pigs.

Detoxification of dilute solutions of diphtheria toxin in fitted tubes may be prevented either by the addition of a solution of normal horse serum[2] to the toxin solution, or by first treating the tubes with dilute serum and subsequent rinsing.

The results in Tables V and VI show this protective action of serum.

A number of possibilities suggest themselves which might account for the disappearance of diphtheria toxin under the conditions which have been described above.

(1) An increase in alkalinity of the solution in the outer compartment, due to the large glass surface, might bring about a decrease in toxicity since toxin is less stable in alkaline than in neutral solution [Moloney and Taylor, 1930].

[1] Up to the moment we have not concerned ourselves with the constituent or constituents in the light petroleum used, which may be responsible for the effect shown in Table IV.

[2] Gelatin [Moloney and Taylor, 1932] is also an effective protecting agent; and no doubt many other proteins exert the same action.

Table V. *Stabilising effect of serum on toxin in tubes coated with paraffin wax.*

No serum		1/27,000 diln. serum		1/9000 diln. serum		1/3000 diln. serum		1/1000 diln. serum	
Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume	Large surface / volume	Small surface / volume
0	20	17	20	19	19	19	21	17	18
0	20	0	20	19	19	21	21	16	20
0	20	13	19	17	19	18	17	19	20

Note. The same effect is observed when glass tubes sterilised with cotton plugs are used in place of tubes coated with paraffin wax.

Table VI. *Effect of previous treatment of fitted tubes with serum on the disappearance of toxin.*

Treatment of tubes	1/2000 diln. toxin No. 461: Large surface / volume	1/2000 diln. toxin No. 461: Small surface / volume
Tubes sterilised with metal caps	19	17
	18	18
Tubes sterilised with cotton plugs	0	18
	0	19
	0	19
Tubes sterilised with metal caps; treated with buffer 20 hrs.; rinsed before adding toxin	22	22
	20	20
	21	21
Tubes sterilised with cotton plugs; treated with buffer 20 hrs.; rinsed 10 times before addition of toxin	16, 0, 0, 8 *v.f.* 0, 0	21, 20, 20, 23, 20, 20
Tubes sterilised with cotton plugs; treated with 1/500 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	18	20
	19	19
	19	21
Tubes sterilised with cotton plugs; treated with 1/8000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	22	22
	20	20
	17	25
Tubes sterilised with cotton plugs; treated with 1/16,000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	0	20
	10	21
	0	22
Tubes sterilised with cotton plugs; treated with 1/32,000 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	0	20
	0	22
	0	22
Paraffined tubes treated with 1/500 dilution serum 20 hrs.; rinsed 10 times before addition of toxin	19	27
	22	27
	17	23

Note. Tests made on the final rinse waters showed that insufficient serum was left in the tubes to exert any protecting action.
v.f. = very faint reaction.

However, measurements made with the hydrogen electrode failed to show any difference in the p_H of solutions in the two compartments of the fitted tubes.

(2) In another communication [Moloney and Taylor, 1930] we have shown that diphtheria toxin may be rendered atoxic by means of oxidising agents. It

is conceivable that the toxin might have been destroyed by oxidation at the glass surface, owing, for example, to adsorbed oxygen or chromic acid. That oxidation is not a probable factor is shown by the following experiment. Dilute toxin solution containing sodium thiosulphate was put into fitted tubes. Examination of the solutions after the tubes had stood for 16 hours at room temperature showed a marked decrease in toxicity of those solutions which had been in contact with the large surface. Sodium thiosulphate was still present in the solutions at the conclusion of the experiment.

(3) Some detoxifying substance might be leached from the surface by the toxin solution. In connection with this possibility we have attempted to leach a detoxifying substance from glass surfaces—previously heated with cotton-wool—using as leaching agents buffered saline and saline to which serum had been added in amount insufficient to prevent the disappearance of toxin in fitted tubes. After some hours' contact with the surface, the leach solutions were used to dilute toxin and the diluted toxin was allowed to stand for 16 hours at room temperature. There was no measurable loss of the toxicity of these solutions. Moreover the tubes which had been leached had approximately the same detoxifying capacity as the untreated tubes.

(4) Toxin might be adsorbed from solution in contact with the relatively large surface. There are three ways in which adsorption might be demonstrated.

(*a*) By showing directly, with the use of animals, that the adsorption complex contains either toxin or immunising substance. This was done by Freund [1930] with diphtheria toxin adsorbed on collodion particles. In our experiments we could not conveniently carry out such a test.

(*b*) By leaching toxin from the surface. It has been shown that toxin adsorbed on aluminium hydroxide [Schmidt, 1931], magnesium hydroxide [Ecker and Wied, 1932], and barium sulphate [Siebenmann] may be recovered by suitable leaching agents. We have attempted to recover toxin from glass and paraffined glass surfaces, which had previously been treated with toxin, by the use of serum solution. There was no evidence that any toxin had been leached off.

(*c*) By showing that the surface after contact with toxin possesses specific combining power for diphtheria antitoxin. Freund [1930] and Eisler [1923] have shown that, with collodion and charcoal respectively, adsorbed diphtheria toxin will combine specifically with antitoxin. In Table VII are summarised the results of experiments in which fitted tubes sterilised with cotton plugs were first treated with a solution of diphtheria toxin, purified by the method of Siebenmann, and then tested for combining power with diphtheria antitoxin.

In these experiments there is definite evidence that diphtheria toxin has been adsorbed. It must be pointed out, however, that this holds only in the case of tubes which had been treated with solutions of purified toxin. Further, the amount of toxin which can be accounted for on the surface by antitoxin-combining power is of the order of 1/500 of the amount which will disappear from a solution in contact with an equivalent area of glass or paraffin wax

Table VII. *Antitoxin-combining power of surface after treatment with toxin.*

Method of treating tubes	Antitoxin-combining power of final rinsing	Concentration of antitoxin and serum added to tubes; 16 hrs. room temperature	Tests on antitoxic solns. from large $\frac{\text{surface}}{\text{volume}}$ compartments
Soln. purified toxin 3 Lf per cc. left in tubes for 20 hrs. at room temp. Rinsed 10 times before addition of anti-toxin	Final rinse water (buffered saline) left in tubes for 3 hrs. No evidence of combining power	1/1000 unit per cc. +1/2000 normal serum	Injected 0·2 cc. of equal amounts of a toxin dilution and antitoxic solution 19 15 15 19 16 21
Fitted tubes not treated with toxin		1/1000 unit per cc. +1/2000 normal serum	0 0 10 *v.f.* 12 *v.f.*

Note. A toxic reaction denotes a decrease in concentration of antitoxin in the solutions tested. *v.f.* = very faint reaction.

surface. In other experiments we have attempted without success to demonstrate antitoxin-combining power of surfaces which had been treated with dilutions of unpurified toxic broth filtrate. None of these was successful.

This failure to demonstrate the specific adsorption of antitoxin by glass previously treated with a solution of unpurified toxin might have been due to the relatively small amount of toxin adsorbed in comparison with the amount of substances other than toxin adsorbed from the toxin broth filtrate.

Disappearance of antitoxin in contact with large surfaces.

In the experiments recorded in Table VII it was necessary to know the effect on solutions of antitoxin of contact with relatively large surfaces. Results in Table VIII show that antitoxin disappears from solution in the outer compartment of the fitted tubes—large surface to volume—which had been

Table VIII. *Disappearance of antitoxin in fitted tubes and protective action of added serum.*

	Antitoxin 1/100 unit per cc.		Antitoxin 1/100 unit per cc. +1/2000 diln. normal serum		Antitoxin 1/200 unit per cc.		Antitoxin 1/200 unit per cc. +1/2000 diln. normal serum	
Treatment of tubes	Large surface/vol. L+/200 tox.	Small surface/vol. L+/200 tox.	Large surface/vol. L+/200 tox.	Small surface/vol. L+/200 tox.	Large surface/vol. L+/300 tox.	Small surface/vol. L+/300 tox.	Large surface/vol. L+/300 tox.	Small surface/vol. L+/300 tox.
Tubes sterilised with metal caps	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Tubes sterilised with cotton plugs	15	0	0	0	15	0	0	0
	17	0	0	0	13	0	0	0
	12	0	0	0	9	0	0	0
	16	0	0	0	—	—	—	—
	18	0	0	0	—	—	—	—
Tubes coated with paraffin wax	13	0	0	0	13	0	0	0
	15	0	0	0	13	0	0	0
	16	0	0	0	12	0	0	0

Note. The antitoxin solutions used were dilutions in buffered saline (p_H 7·02) of antitoxic horse serum 350 units per cc. which contained no added preservative.

sterilised with cotton plugs or which had been coated with paraffin wax, and that this disappearance is prevented when sufficient serum is first added to the antitoxin solution.

Conclusions.

1. Diphtheria toxin disappears from solution in contact with a sufficiently large surface of paraffin wax, or of glass which has been first in contact with light petroleum, or of glass which has been heated in an air-oven in close proximity to cotton-wool or filter-paper for one hour at 180° (tubes plugged with cotton-wool or with filter-paper). Glass, cleaned with chromate-sulphuric acid solution and heated in the air-oven at 180° for one hour, has relatively little effect on toxin solutions in contact with it.

2. Similarly diphtheria antitoxin disappears from solution in contact with a paraffin wax surface or with glass previously exposed to heated cotton-wool.

3. It is probable that the disappearance of toxin and antitoxin from solutions in contact with paraffin wax and glass is due to adsorption on the solid surfaces.

REFERENCES.

Ecker and Wied (1932). *J. Immunol.* **22**, 61.
Eisler (1923). *Biochem. Z.* **135**, 416.
Freund (1930). *Proc. Soc. Exp. Biol. Med.* **28**, 65.
Glenny, Pope, Waddington and Wallace (1925). *J. Path. Bact.* **28**, 471.
Moloney and Taylor (1930). *Proc. Roy. Soc. Can.* (Sec. V), **24**, 127.
—— —— (1932). *Amer. J. Pub. Health*, **22**, 38.
Pope (1927). *J. Path. Bact.* **31**, 301.
Schmidt (1931). *Ann. Inst. Pasteur*, **46**, 202.
Siebenmann. (In Press.)

CCVIII. A CELLULASE FROM THE SYMBIOTIC INTESTINAL FLAGELLATES OF TERMITES AND OF THE ROACH, *CRYPTOCERCUS PUNCTULATUS*.

By WILLIAM TRAGER.

From the Department of Tropical Medicine, Harvard University Medical School, Boston, Massachusetts.

(*Received August 16th, 1932.*)

The number of organisms able to utilise cellulose as the sole source of carbohydrate is small, and the number from which it has been possible to isolate a cellulase is smaller still. Karrer *et al.* [1923; 1924; 1925] have done extensive work with a cellulase from the intestinal juice of the vineyard snail, as have Pringsheim and Bauer [1928] and Pringsheim and Leibowitz [1928] with a very similar enzyme obtained from malt. Boynton, Lyman and Miller [1927] were able to demonstrate cellulase somewhere in the anterior portion of the shipworm, *Bankia setacea*. Jewell and Lewis [1918] found lichenase in the alimentary system of each of twenty species of invertebrates. Since lichenin is a form of cellulose [Karrer, Joos and Staub, 1923] the active extracts from these animals would probably also have hydrolysed cellulose. Pringsheim [1912; 1919] showed long ago the presence of a cellulase in cellulose-fermenting bacteria, but this enzyme loses its activity very soon after the death of the bacteria, and has never been obtained in a cell-free condition.

Of all the cellulose-feeding organisms there are few whose diet contains as high a percentage of cellulose as does that of termites. Cleveland [1923; 1924] has shown that these insects can live for long periods of time on a diet of almost pure cellulose, if, and only if, their normal complex intestinal fauna has not been removed. This fauna consists mainly of representatives of two orders of flagellates: the polymastigotes and the hypermastigotes. The great majority of these intestinal protozoa ingest cellulose particles and apparently digest them. They might be presumed to contain a cellulase.

Most termites, on account of their small size, do not furnish very good material for the preparation of enzyme extracts. Recently, however, it has been discovered that the large wood-feeding roach, *Cryptocercus punctulatus*, possesses a fauna of intestinal flagellate symbionts very similar in all respects to those of termites [Cleveland, 1930]. These roaches, obtained through the kindness of Dr L. R. Cleveland, proved to be an excellent source of the enzyme, cellulase, and they were used for most of the work reported in this paper. The

presence of the enzyme has also been demonstrated in the termites, *Reticulitermes flavipes* and *Termopsis angusticollis*, as well as in pure cultures of one of the protozoa from the latter.

Action of the cellulase. Proof that it is secreted by the intestinal protozoa.

It was a simple matter to show that a cellulase was present in the alimentary tract of *Cryptocercus*. Only a single typical experiment need be detailed. The guts of twenty roaches were ground in a mortar with sand to a brown paste. This was diluted with distilled water to 25 cc. giving a light brown suspension, which, after filtration through fine filter-paper, became almost white. The extract, covered with toluene, was dialysed in a collodion bag against running water for 18 hours to rid it of all reducing substances. Two tubes were then prepared, one containing 1 cc. of the extract, the other 1 cc. of the extract with a small amount of reprecipitated, powdered cellulose. The liquid in both was covered with toluene and the tubes were incubated at 35·5°. Tests were made for glucose with Benedict's reagent.

	Benedict's test after	
Tube contents	4 hours	24 hours
1 cc. extract	–	–
1 cc. extract + cellulose	+	+ + +

– means test negative.

An active extract could also be obtained by grinding up the gut of a single roach, diluting to 5 cc. and centrifuging.

In several cases, the digest, after the action of the enzyme on the cellulose had ceased, was evaporated to dryness over a water-bath, and the residue taken up in phenylhydrazine-sodium acetate solution. On boiling, typical glucosazone crystals were obtained. In one experiment these were filtered off, washed and dried, and the melting-point found to be 202–204° (uncorr.).

It next appeared important to determine whether the cellulase was secreted by the protozoa, or by the host, or by both. Insect enzymes are typically produced in the midgut and in the salivary glands [Abbott, 1926; Wigglesworth, 1927]. The flagellates of *Cryptocercus* are present only in the hind-gut [Cleveland, Hall and Sanders, unpublished]. The following experiment was accordingly performed. The salivary glands, fore- and mid-guts, and hind-guts of 5 roaches were carefully removed, and a separate extract made from each. The salivary glands, after having been ground up, were diluted with water to 3 cc., the fore- and mid-guts to 3 cc., and the hind-guts to 15 cc. The extracts were partly cleared by centrifuging.

	Benedict's test after	
Tube contents	1 day	3 days
1 cc. salivary gland extract, 0·5 cc. 95 % alcohol	–	–
Same with cellulose	–	–
1 cc. fore- and mid-gut extract, 0·5 cc. 95 % alcohol	–	trace
Same with cellulose	–	trace
1 cc. hind-gut extract, 0·5 cc. 95 % alcohol	–	–
Same with cellulose	+ +	+ +

The enzyme is thus present only in the hind-gut, a fact which indicates its protozoan origin.

Still more conclusive proof of the protozoan origin of the enzyme was obtained from the following experiment, done in conjunction with Dr L. R. Cleveland. A group of roaches was fed for 1½ months on nearly pure cellulose (Whatman filter-paper No. 40). The roaches were then divided into three groups. One group (*A*) received no further treatment. A second (*B*) was defaunated by keeping the insects at a temperature of 34° for 6 hours, a procedure which in no way injures the roach. The third group (*C*) was defaunated by oxygenation at 4 atmospheres for 2 hours [Cleveland, 1925]. An extract from a roach of group *A* was oxygenated at the same time with the insects of group *C*. The latter, after treatment, were fed on the alimentary canal material of roaches killed and defaunated by keeping them at a temperature of − 10° for 1 hour. This was done so that the group *C* roaches could be reinfected by any bacteria that might have been killed by the oxygen treatment (bacteria are generally very resistant to low temperatures) and thereby have a normal intestinal flora. Extracts were made from each group, using the gut of one roach diluted to 5 cc. and centrifuged, with results as summarised below.

No.	Tube contents	Benedict's test after 1 day	2 days	3 days
1	1 cc. from *A*, 0·5 cc. 95 % alcohol	−	0	0
2	Same as 1 with 0·25 cc. cellulose suspension	+ +	0	0
3	Same as 1 oxygenated 2 hrs., 4 atm.	−	0	0
4	Same as 2 oxygenated 2 hrs., 4 atm.	+ +	0	0
5	1 cc. from *B*, 1 day after treatment, 0·5 cc. 95 % alcohol	−	−	−
6	Same as 5 with 0·25 cc. cellulose suspension	−	−	−
7	1 cc. from *C*, 2 days after treatment, 0·5 cc. 95 % alcohol	−	−	−
8	Same as 7 with 0·25 cc. cellulose suspension	−	−	−

0 means not tested.

It is evident that regardless of the method of defaunation used, the enzyme disappears coincidently with the disappearance of the protozoa, although the treatments which destroy the protozoa injure neither the enzyme extract nor the roach. The roach itself secretes no cellulase, a fact which is very important with respect to the symbiotic nature of the flagellates.

Still further proof that the cellulase is secreted by the flagellates was furnished by the action of an extract prepared from cultures of *Trichomonas termopsidis*, a xylophagous, intestinal flagellate of the termite, *Termopsis angusticollis*. This organism, now in its 22nd subculture, has been cultivated for almost 2 years in an artificial medium containing finely powdered cellulose[1]. The cultures are contaminated by only one species of bacterium, a small bacillus which actively ferments dextrose but is incapable of attacking cellulose. The contents of 20 culture-tubes were centrifuged, and the small mass of protozoa thus obtained was ground with sand, diluted to 2 cc. and cleared by centrifuging. The results were:

Tube contents	Benedict's test after 2·5 hrs.
1 cc. extract, 0·5 cc. phosphate buffer (p_H 5·3), 0·5 cc. water	−
1 cc. extract, 0·5 cc. phosphate buffer (p_H 5·3), 0·5 cc. cellulose suspension	+ +

[1] Full details of the cultivation will be given in a later paper.

Presence of the cellulase in termites.

The guts of 100 *Reticulitermes flavipes* were ground with sand and diluted with water to 3 cc. The enzyme was adsorbed on aluminium hydroxide and eluted with 3 % KH_2PO_4.

Tube contents	Benedict's test after 5 hours
0·5 cc. extract, 0·5 cc. buffer (p_H 5·3), 0·25 cc. cellulose suspension	+ +
Same but without cellulose	–

The gut of a single *Termopsis angusticollis* was ground with sand and diluted with water to 2 cc. This extract also showed typical enzyme action.

Properties and partial purification of the cellulase.

The properties of this protozoan cellulase have been studied in some detail, mainly to provide a basis for comparison of this enzyme with cellulases from other organisms, notably snail-cellulase and malt-cellulase.

Very early in the course of the work it was noted that the physical state of the cellulose had much to do with the ease with which it was attacked by the enzyme. Thus an extract of 25 *Cryptocercus* guts dialysed 24 hours and made up with alcohol and distilled water to 25 cc. containing 30 % alcohol gave these results (incubated at room temperature):

	Benedict's test after		
Tube contents	1 day	2 days	3 days
5 cc. extract, ground filter-paper	–	–	+ +
5 cc. extract, cellulose suspension	+ +	+ + +	0
5 cc. extract	–	–	–

The extract of a single roach gut diluted to 6 cc. with distilled water gave:

Tube contents	Benedict's test after 1 day at room temperature
1 cc. extract, cellulose suspension	+ + +
1 cc. extract, filter-paper	+
1 cc. extract	–

The material referred to as cellulose suspension was prepared by v. Weimann's method in accordance with the directions given by Pringsheim and Bauer [1928]. 1 g. of pure filter-paper was dispersed in a solution of 50 g. of lithium chloride in 50 cc. of water at a temperature of 160°. The solution so obtained was poured into about 600 cc. of cold water, whereupon the cellulose precipitated out in a very fine flocculent form. It was washed and centrifuged about 9 times with water. The final residue was taken up in distilled water to give a suspension having about 6 to 8 mg. of cellulose per cc. The surface of the cellulose exposed to enzyme action by such a preparation is much greater than that exposed by even finely powdered filter-paper and the micellar structure is probably also different. Karrer, Schubert and Wehrli [1925] have shown that both these factors are important with respect to the degree of hydrolysis of

various cellulose preparations by snail-cellulase. Any form of treated cellulose, although chemically identical with native cellulose, was more easily hydrolysed than the latter.

The enzyme concentration also affected the extent of hydrolysis. A series of tubes was prepared in which a watery extract of the cellulase was diluted 1–4, 1–8, 1–16, *etc.* to 1–128 with phosphate buffer at a p_H of 5·3. The results were:

Dilution of extract	Roach units per cc.	Benedict's test after 2 hours
1–4	5/6	+ + + + +
1–8	5/12	+ + + +
1–16	5/24	+ + +
1–32	5/48	+ +
1–64	5/96	+
1–128	5/192	–
1–4 (no cellulose)	5/6	–

One roach unit is the extract from one roach. This term will be used to give an approximate idea of the concentration of material in the various extracts. It has meaning only when preparations from one and the same extract are being compared. Some quantitative results indicating the effect of concentration of enzyme on the time course of the reaction, cellulose → glucose, are given in a later portion of this paper.

The cellulase was inactivated by 10 minutes' immersion in a boiling water-bath, as well as by 1 hour's at 60°. The extracts were treated first and then were mixed with cellulose suspension and allowed to act on it at a temperature of 35° for periods of time up to 24 hours.

It has been possible partially to purify the enzyme extracts. The original watery preparations are always smooth brown suspensions which pass through coarse filter-paper unchanged. Filtration of such an aqueous extract through a Seitz asbestos filter gives a clear colourless filtrate devoid of enzyme. If, however, enough alcohol is first added to the extract to give a 30 % concentration, and the mixture is kept about a day, subsequent filtration through a Seitz filter gives a clear, slightly yellowish filtrate, which does contain the enzyme, although in not nearly as high a concentration as in the unfiltered material. In watery extracts the cellulase appears not to pass through a collodion membrane, as shown below.

Tube contents	Benedict's test after 2 hours	Benedict's test after 1 day
1 cc. extract, 1 cc. phosphate buffer (p_H 5·3), 0·5 cc. cellulose suspension	+ + +	+ + +
1 cc. extract in a collodion bag immersed in 1 cc. phosphate buffer (p_H 5·3) with 0·5 cc. cellulose suspension	–	–

In this respect this cellulase seems to differ from the lichenase of Karrer, Staub and Joos [1924, 1] which did act on lichenin through a collodion membrane. In the present instance the collodion bag was made from Merck's U.S.P. collodion, allowed to dry 10 minutes, fixed in 95 % alcohol and washed in water. The membrane used by Karrer, Staub and Joos may have been prepared in a different manner.

The enzyme was not adsorbed on Merck's powdered animal charcoal, but all the brown material present in the extract could be removed in this way. The cellulase could be adsorbed completely by aluminium hydroxide and eluted with 3 % KH_2PO_4, thus again behaving like snail-cellulase [Karrer, Staub and Joos, 1924, 1]. It could also be precipitated with acetone and dried with ether. The experimental details follow.

One hundred roach guts were ground with sand and diluted with distilled water to 30 cc. The mixture was centrifuged and the supernatant liquid dialysed against running water for 19 hours. Three 5 cc. portions of the extract were now subjected to three different treatments. Part 1 was diluted with distilled water to make 10 cc. To part 2, 5 cc. of acetone were added, giving a brown precipitate which was filtered off, washed twice with ether and allowed to air-dry. Part 3 was treated with aluminium hydroxide paste and filtered. The clear colourless filtrate may be designated as part 3*a*. The residue was washed thrice with 10 cc. of 3 % KH_2PO_4, the final preparation (3) being a colourless, slightly turbid liquid. Test- and control-tubes were now set up in this manner: each control-tube contained 3 cc. of extract, 2 cc. phosphate buffer (p_H 5·3) and 1 cc. of distilled water; each test-tube held the same, except that 1 cc. of cellulose suspension replaced the 1 cc. of water. In the case of part 2 the dry powder was suspended in 6 cc. of water to give a concentration of 1·3 mg. per cc. The material in the tubes was covered with toluene. The tubes were incubated for about 2 hours at 35·5°, and the glucose concentration in each was then determined by the Willstätter-Schudel [1918] iodimetric method [modified by Kline and Acree, 1930]. The results were:

Part No.	Mg. glucose per cc.
1	4·3
2	0·2
3	0·8
3*a*	0

The method of precipitation and drying involves a considerable loss. Adsorption on aluminium hydroxide and elution with KH_2PO_4 also occasion a loss of enzyme due to the incomplete nature of the elution. The activity of the purified enzyme is, however, sufficiently great for its use in quantitative studies.

Effect of enzyme concentration and of temperature.

Because of the restricted amount of material available, quantitative work with this protozoan cellulase has necessarily been limited. Enough results have been obtained to indicate the type of reaction and the way in which it is affected by changes in enzyme concentration and in temperature. The reaction was followed in all cases by the titration of samples, removed at intervals, by the modified Willstätter-Schudel iodimetric method, using 2 cc. of 0·1 *N* iodine solution, 3 cc. of 0·1 *N* NaOH and a 5 cc. Folin burette for the thiosulphate.

The protozoa from 10 *Cryptocercus* were washed and centrifuged in a balanced salt solution in which they could live for long periods of time. The mass

so obtained was ground with sand, diluted to 10 cc. with distilled water and centrifuged. 5 cc. of the extract were mixed with 25 cc. of buffer (p_H 5·3) and 5 cc. of cellulose suspension. The mixture was kept at 35° and 1 cc. samples were removed. The data are plotted in Fig. 1.

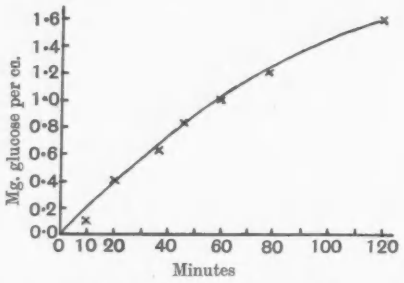


Fig. 1. Hydrolysis of cellulose by an unpurified extract at 35°.

In another experiment the washed protozoa from 30 roaches were similarly extracted. The extract was treated with aluminium hydroxide paste and the enzyme eluted with 30 cc. of 3 % KH_2PO_4. Thus 1 cc. of this preparation contained 1 roach unit. Tubes were set up containing cellulose suspension and enzyme appropriately diluted with phosphate buffer of p_H 5·3. The liquid in each case was covered with toluene and 1 cc. samples were taken. The results are shown graphically in Fig. 2.

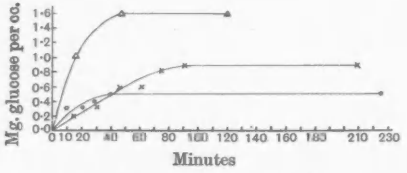


Fig. 2. Effect of enzyme concentration on the hydrolysis of cellulose by a purified extract at 33·5°.

—o— 0·2 roach unit per cc.
—x— 0·5 roach unit per cc.
—△— 0·75 roach unit per cc.

With yet another similarly purified extract, these results were obtained, keeping the concentration of enzyme the same:

Temperature	Mg. glucose per cc. after 2 hours
16°	0·3
21°	0·4
26°	0·7
34°	0·2

A temperature of 26° was thus more favourable, over a period of 2 hours, than any of the lower or higher temperatures tried. For a real analysis of the

temperature effect, it will be necessary to compile a family of curves showing the rates of hydrolysis at various temperatures. The velocity constant of the hydrolysis at each temperature could then be calculated (assuming that the reaction follows the unimolecular law) and the relation of these constants to temperature found.

Cellobiase.

Some cellobiose was prepared from the octa-acetate according to the method of Peterson and Spencer [1927]. It was dissolved in phosphate buffer of p_H 5·3 at a concentration of 0·01 g. per cc., and used in the following experiment.

Washed protozoa from 30 roaches were ground and the mixture centrifuged. The supernatant liquid was made up to 10 cc. with water, giving extract 1, with 3 roach units per cc. 7 cc. of 1 were treated with aluminium hydroxide and the enzyme eluted with 10 cc. of 3 % KH_2PO_4 giving the purified extract 2, with 2 roach units per cc. Two tubes were prepared. One (*A*) contained 2 cc. of 1, 1 cc. of 3 % KH_2PO_4 and 3 cc. of the cellobiose solution to give a mixture having 1 roach unit and 0·005 g. of cellobiose per cc. The other (*B*) held 3 cc. of 2 and 3 cc. of the cellobiose solution giving a mixture with 1 roach unit (purified) and 0·005 g. of cellobiose per cc. The contents of both tubes were covered with toluene. The tubes were incubated at 30·1°. At intervals 1 cc. samples were removed and titrated iodimetrically. The data are summarised in Table I. The figures in column 6 were obtained in the following way. 5 mg.

Table I.

(1) Tube No.	(2) Time (minutes)	(3) Milliequiv. I	(4) Milliequiv. $Na_2S_2O_3$	(5) Total milli-equiv. I used	(6) (see text)	(7) % hydrolysis
A	0	0·1960	0·1466	0·0494	0·0292	0
	15	0·2940	0·2399	0·0541	0·0339	16·2
	30	0·2940	0·2380	0·0560	0·0358	22·8
	45	0·2940	0·2323	0·0617	0·0415	42·7
	135	0·2940	0·2152	0·0788	0·0586	100
B	0	0·2940	0·2599	0·0341	0·0292	0
	15	0·2940	0·2599	0·0341	0·0292	0
	30	0·2940	0·2570	0·0370	0·0321	10
	60	0·2940	0·2513	0·0427	0·0378	30
	120	0·2940	0·2456	0·0484	0·0435	50

of cellobiose react with 0·0292 milliequiv. of iodine. This number was accordingly subtracted from the total milliequiv. used at zero time to give the milliequiv. of iodine used by the mixture itself apart from the cellobiose. This latter figure was then in each case subtracted from the total milliequiv. used to give the numbers in column 6. The reducing power of the mixture itself was presumably due to the presence of glucose and was very much less (almost negligible) in the purified extract than in the unpurified. The percentage hydrolysis was obtained from Table II, constructed after a similar table used by Pringsheim and Bauer [1928]. Fig. 3 illustrates the course of the hydrolysis in tubes *A* and *B*.

About 18 hours after the start of the reaction, the remaining liquid in tube *A* was evaporated to dryness and the residue taken up in phenylhydrazine-

Table II.

% hydrolysis	Mg. cellobiose	Milliequiv. I	Mg. glucose	Milliequiv. I	Total milliequiv.
0	5	0·0292	0	0	0·0292
10	4·5	0·0263	0·525	0·0058	0·0321
20	4	0·0233	1·05	0·0117	0·0350
30	3·5	0·0204	1·575	0·0175	0·0379
40	3	0·0174	2·1	0·0233	0·0407
50	2·5	0·0146	2·625	0·0291	0·0437
60	2	0·0117	3·15	0·0350	0·0467
70	1·5	0·0087	3·675	0·0408	0·0495
80	1	0·0058	4·2	0·0466	0·0524
90	0·5	0·0029	4·725	0·0525	0·0554
100	0	0	5·25	0·0583	0·0583

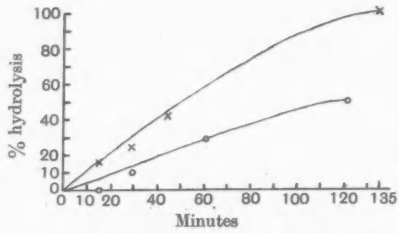


Fig. 3. Hydrolysis of cellobiose at 30·1°.
—×— Unpurified extract (tube *A*).
—o— Same extract purified (tube *B*).

sodium acetate solution. Glucosazone was obtained on boiling and was filtered off while the mixture was hot. The filtrate, on cooling, gave no crystals of cellobiosazone. The remaining liquid in tube *B* was treated in the same manner. In this case both glucosazone and an osazone soluble in hot water (cellobiosazone) were formed.

This protozoan cellulase thus contains, like malt-cellulase and snail-cellulase, a cellobiase.

Summary.

A cellulase has been demonstrated in the symbiotic intestinal flagellates of *Cryptocercus punctulatus*, *Reticulitermes flavipes* and *Termopsis angusticollis*.

The enzyme has also been extracted from one of these flagellates, *Trichomonas termopsidis*, grown for 2 years in pure culture.

The active extract hydrolyses cellulose to glucose. It also hydrolyses cellobiose to glucose. Possibly two enzymes are concerned, a cellulase proper and a cellobiase.

The cellulase complex can be adsorbed on aluminium hydroxide and eluted with 3 % KH_2PO_4. In this and in several other respects it resembles snail-cellulase and malt-cellulase.

Data are presented indicating the way in which the hydrolysis of cellulose by this protozoan cellulase is affected by enzyme concentration and by temperature.

Acknowledgment is due to Dr L. R. Cleveland under whom this work was done, and also to Dr A. E. Navez of the Physiology Department, Harvard University.

REFERENCES.

Abbott (1926). *J. Exp. Zool.* **44**, 219.
Boynton, Lyman and Miller (1927). *J. Biol. Chem.* **75**, 613.
Cleveland (1923). *Proc. Nat. Acad. Sci.* **9**, 424.
—— (1924). *Biol. Bull.* **48**, 289.
—— (1925). *Biol. Bull.* **48**, 309.
—— (1930). *Anat. Rec.* **47**, 293.
—— Hall and Sanders (in manuscript).
Jewell and Lewis (1918). *J. Biol. Chem.* **33**, 161.
Karrer, Joos and Staub (1923). *Helv. Chim. Acta*, **6**, 800.
—— Schubert and Wehrli (1925). *Helv. Chim. Acta*, **8**, 797.
—— Staub and Joos (1924, 1). *Helv. Chim. Acta*, **7**, 144.
—— —— —— (1924, 2). *Helv. Chim. Acta*, **7**, 154.
Kline and Acree (1930). *Bureau of Standards J. Res.* **5**, 1063.
Peterson and Spencer (1927). *J. Amer. Chem. Soc.* **49**, 2822.
Pringsheim (1912). *Z. physiol. Chem.* **78**, 266.
—— (1919). Die Polysaccharide. (Berlin.)
—— and Bauer (1928). *Z. physiol. Chem.* **173**, 188.
—— and Leibowitz (1928). Die Methodik der Fermente III, 902. (Thieme, Leipzig.)
Wigglesworth (1927). *Biochem. J.* **21**, 51.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.

CCIX. WOOD CELLULOSE.

IV. FURTHER INVESTIGATION OF THE "RESISTANT PORTION" OF THIRIENA PULP.

By DAVID JAMES BELL.

From the Chemistry Research Laboratories of the United College of St Salvator and St Leonard, The University of St Andrews.

(*Received September 7th, 1932.*)

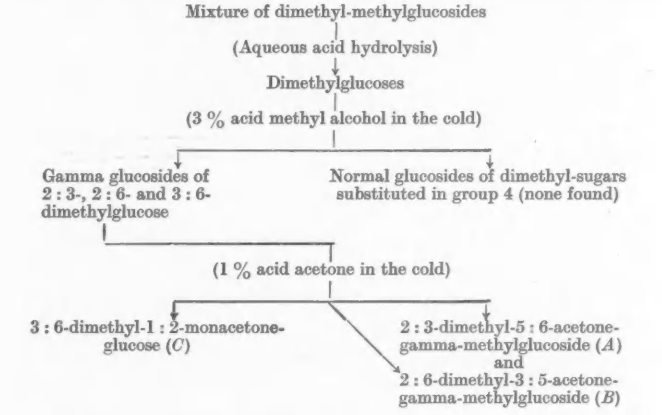
The discovery by the author of the existence within wood celluloses of fractions resistant both to complete etherification and to hydrolysis has already been described in this *Journal* [1932, 1, 2, 3]. The causes underlying this peculiar inactivity of certain of the free hydroxyl groups attached to the glucose residues composing the resistant material could not be explained on grounds either of physical condition or of the positions occupied by these groups. Acetylation of the methylated resistant portions, which had compositions approximating to that of a dimethylcellulose, was easily effected by Barnett's process [1921], when it was found that extensive depolymerisation had accompanied the acylation of the free hydroxyl group. Cleavage of these products, by the action of methyl alcohol containing hydrogen chloride, could now be readily carried out, and it was found that the cleavage products consisted of a mixture of at least five partially methylated glucoses (as the methylglucosides). Individually these substances were 2 : 3 : 6-trimethylglucose; 2 : 3-dimethylglucose; 2 : 6-dimethylglucose; 3 : 6-dimethylglucose; 6-monomethylglucose. Other isomerides of trimethyl- and dimethyl-glucose were absent. No monomethylglucose other than the 6-derivative could be detected, although the absence of isomerides was not proved.

The extremely scanty information at present available regarding the structure of polysaccharides narrowed down to a small range the methods of investigation applicable to the present problem. It was decided to effect a comparison of the dimethyl-sugars produced on hydrolysis of the resistant portions with those derived from the partially methylated non-resistant fractions. To this end a specimen of Thiriena pulp was methylated to a methoxyl content of 27·9 %. Cleavage of the methylate yielded 29·2 % of resistant material, showing a methoxyl content of 19·1 %. Hence, calculation shows that the remaining 70·8 % of non-resistant material had, in the methylate, a methoxyl content of 31·3 %, a value closely similar to the methoxyl content of the fully methylated resistant portion of Thiriena pulp, namely 30·3 %.

The resistant material, after methylation and acetylation, displayed properties identical with those of previous preparations. The mixed glucosides derived from the non-resistant portion contained about 50 % of the dimethyl compounds: after removal of trimethyl derivatives by extraction of an aqueous solution with chloroform, the dimethyl-methylglucosides were purified by the process of extraction from strong potassium carbonate solution [Bell, 1932, 1] and finally distilled in a high vacuum. The resulting colourless syrup was employed in the comparative experiment, the material from the resistant portion having been previously investigated and briefly reported on [Bell, 1932, 3].

The methods employed were those devised by Rutherford [1932], and Oldham and Rutherford [1932], which have been applied to the hydrolysis products of methylated starch by Irvine and Rutherford [1932]. Since complete condensation of the free sugars with methyl alcoholic hydrogen chloride took place in the cold, neither batch of material contained derivatives of glucose with the 4th position substituted. The method of investigation of the mixed glucosides is summarised diagrammatically, as follows.

A. *Estimation of the amount of substitution of the fourth group in the glucose chains. Estimation of the amount of 3 : 6 dimethylglucose.*


Mixture of dimethyl-methylglucosides
(Aqueous acid hydrolysis)
Dimethylglucoses
(3 % acid methyl alcohol in the cold)
Gamma glucosides of 2 : 3-, 2 : 6- and 3 : 6-dimethylglucose
Normal glucosides of dimethyl-sugars substituted in group 4 (none found)
(1 % acid acetone in the cold)
3 : 6-dimethyl-1 : 2-monacetone-glucose (C)
2 : 3-dimethyl-5 : 6-acetone-gamma-methylglucoside (A) and 2 : 6-dimethyl-3 : 5-acetone-gamma-methylglucoside (B)

(C) (A) (B)

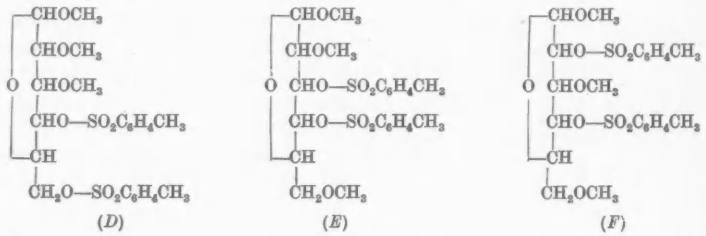
Methoxyl estimation of the mixture indicates the proportion of 3 : 6 dimethylglucose.

B. *Estimation of the amount of 2 : 3-dimethylglucose by determination of the amount of free hydroxyl in group 6.*

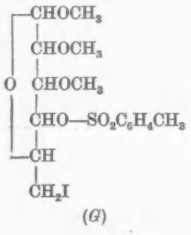
Mixed dimethyl-methylglucosides

↓ (*p*-toluenesulphonyl chloride in pyridine)

2 : 3-dimethyl-4 : 6-di-*p*-toluenesulphonyl-methylglucoside (*D*)
2 : 6 „ 3 : 4 „ „ (*E*)
3 : 6 „ 2 : 4 „ „ (*F*)



(The above mixture was heated with sodium iodide in acetone whereby the *p*-toluenesulphonyl group in position 6 (*D*) was substituted by iodine with the formation of (*G*). Iodine estimation of the product therefore indicates the amount of 2 : 3-dimethylglucose.)



2 : 3-dimethyl-4-*p*-toluenesulphonyl-6-iodo-methylglucoside

The result of the comparison was striking, for, although the same three individual dimethylglucoses were obtained in both cases, their relative proportions were very different. The three partially methylated glucoses, 2 : 3-dimethylglucose, 2 : 6-dimethylglucose, 3 : 6-dimethylglucose, were in the following relative proportions:

	Portion of the wood cellulose	
	Resistant	Non-resistant
Dimethylglucose	%	%
2 : 3	4·4	20·7
2 : 6	58·6	61·3
3 : 6	37·0	18·0

Experimental.

Tabulated details of the condensation with 1 % acid acetone of the mixtures of the γ-glucosides.

Source of material	Resistant	Non-resistant
Amount of glucosides g.	1·721	1·000
Yield of acetone condensation product g.	1·589	0·862
Yield %	80·0	73·8
Mean OMe (2 determinations) %	31·6	33·6
3 : 6-Dimethylglucose found %	37	18
Theoretical OMe for 3 : 6-dimethyl-1 : 2-monoacetoneglucose		25·0 %
Theoretical OMe for monoacetonedimethyl-methylglucoside		35·5 %

Details of the treatment of the normal glucosides with *p*-toluenesulphonyl chloride are appended. The yields of the products were not good; opportunity did not permit of retreatment of the unreacted materials.

Acylation of the mixtures of dimethyl-methylglucosides.

Source	Resistant	Non-resistant
Amount of starting material g.	2·50	5·47
Yield of di-*p*-toluenesulphonates g.	4·06	8·22
Yield %	68·0	63·2
OMe content %	17·2	17·7
Theoretical OMe for dimethyldi-*p*-toluenesulphonyl-methylglucoside		17·5 %

The estimation of the 2 : 3-dimethylglucose.

Amount of material used g.	3·50	4·46
Yield, after NaI treatment g.	3·09	4·32
2 : 3-Dimethylglucose (by iodine estimation) present %	4·4	20·7
Yield of residue, after $AgNO_3$ treatment g.	3·06 (Theoretical)	3·56 (Theoretical)

Discussion.

As Irvine and Rutherford [1932] found in the case of partially methylated starch, derivatives having the 6th position etherified preponderate in the mixture of dimethylglucoses. In neither case now under consideration were the relative proportions of the different sugar derivatives of the same magnitude as found in starch. Further, although the proportion of 2 : 6-dimethylglucose is the same, whether derived from the resistant or the hydrolysable portions of the wood cellulose, quite marked differences exist between the relative amounts of the 2 : 3- and the 3 : 6-derivatives, indicating that etherification first attacks the 6th group in both cases; in the two fractions of the wood cellulose methylation of groups 2 and 3, together, must pursue different courses when 6 is unsubstituted.

Since 2 : 3 : 6-trimethylglucose is obtained as a cleavage product of the resistant portion, a part of that material must be composed of the ubiquitous polysaccharide unit, the 1 : 5- (or 1 : 4-) anhydroglucose chain. There is at present no evidence that the units giving rise to the dimethylated sugars are not similarly constituted. There must, however, be some peculiar steric effect

produced by the arrangement of the units, since these unetherifiable hydroxyl groups can be easily acetylated. This steric effect would seem, also, to be a property of the fundamental groupings, as the resistant properties persist after depolymerisation of the intact cellulose.

SUMMARY.

A fundamental distinction has been demonstrated between the glucose units composing the resistant and the non-resistant portions of a wood cellulose. Comparison of the three dimethylglucoses (2 : 3-, 3 : 6-, 2 : 6-) derived from the fully methylated resistant portion, and from the non-resistant residue methylated to the same extent as the resistant material, showed that they were formed in different relative proportions, indicating that etherification did not pursue the same course in the two portions of the wood cellulose.

The author is greatly indebted to Sir J. C. Irvine for the interest which he has displayed in this work, and to Mr Walter Caldwell for much practical assistance.

REFERENCES.

Barnett (1921). *J. Soc. Chem. Ind.* **40**, 8 T.
Bell (1932, 1). *Biochem. J.* **26**, 590.
—— (1932, 2). *Biochem. J.* **26**, 598.
—— (1932, 3). *Biochem. J.* **26**, 609.
Irvine and Rutherford (1932). In the Press.
Oldham and Rutherford (1932). *J. Amer. Chem. Soc.* **54**, 366.
Rutherford (1932). Private communication.

CCX. CARBOHYDRATE METABOLISM IN HUMAN TRYPANOSOMIASIS.

By ARTHUR WORMALL[1].

From the Human Trypanosomiasis Institute, Entebbe, Uganda.

(*Received September 9th, 1932.*)

During recent years much attention has been directed to the study of blood-sugar changes during trypanosomiasis in animals such as the guinea-pig, rat and rabbit, and it has been considered by many that the changes noted play a significant rôle in the pathogenesis of this disease. The observations of Schern [1925] showed that the life of trypanosomes *in vitro* could be prolonged appreciably by the addition of dextrose or laevulose, and that the serum and the liver of normal animals could reactivate trypanosomes which had almost become motionless, whereas the serum and liver of animals which were in the last stages of trypanosomiasis had much less reactivating power. This author concluded that in trypanosomiasis the blood-sugar and the liver-sugar are gradually used up by the trypanosomes and that a very important pathogenic action of the trypanosomes is a disturbance of the power of the liver to store glycogen, accompanied by a significant hypoglycaemia [*cf.* also review by Schern, 1927]. The same author [1928] found that rabbits and a horse infected with *T. equinum* sometimes showed a marked hypoglycaemia and at other times a hyperglycaemia. Scheff [1928; 1929], as a result of experiments with rats, brought forward similar evidence and concluded that the consumption of sugar and oxygen by the trypanosomes plays a very important rôle in the metabolic disturbance of the host; this disturbance of normal metabolic processes and a state of partial asphyxia are followed, according to Scheff, by an acidosis which contributes to a diminution in the reaction capacity of the host.

Other authors have recorded a hypoglycaemia in rats, mice, guinea-pigs and similar animals infected with trypanosomes, but they are not in complete agreement as to the significance of the phenomenon. Regendanz and Tropp [1927] found that a marked hypoglycaemia exists in rats infected with trypanosomes only in the premortal stages, and conclude that death in these infected rats is not due to a lack of sugar. Regendanz [1929] similarly found that the decreases in the blood-sugar of monkeys infected with *T. rhodesiense* did not run parallel with the number of parasites in the blood. Fenyvessy [1926], Cordier [1927], Knowles and Das Gupta [1928], and Fenyvessy and Scheff [1930], amongst other authors also record a hypoglycaemia in trypanosomiasis of guinea-pigs, rats, *etc.* Dubois and Bouckaert [1927] and Bruynoghe,

[1] Seconded from the Department of Physiology, the University of Leeds.

Dubois and Bouckaert [1927] reach the conclusion that the hypoglycaemia is of little or no clinical significance, but Dubois [1928] considers that in certain acute cases of trypanosomiasis in guinea-pigs and mice, death is due to this lowering of the blood-sugar. Zotta and Radacovici [1929], using guinea-pigs infected with *T. brucei*, obtained marked variations in blood-sugar with a constant premortal hypoglycaemia; these authors, however, state that the latter phenomenon is not specific for trypanosomiasis but occurs in a large number of diseases. Linton [1930], working with rats infected with *T. equiperdum*, observed a normal blood-sugar value until a very late period of the disease; the liver-glycogen was found to be markedly decreased. The same author [1929] had previously recorded a normal blood-sugar in rats infected with *T. lewisi*. The relatively enormous sugar consumption by trypanosomes *in vitro* has been well demonstrated by Yorke, Adams and Murgatroyd [1929], who showed that 0·25 cc. of heavily-infected mouse-blood (*T. rhodesiense* infection) when added to 5 cc. of sheep-serum to which glucose had been added caused the disappearance of between 2 and 2·5 mg. of glucose in one hour.

Other authors have investigated the question of excess lactic acid production in trypanosomiasis. Kligler and Geiger [1928] found that in this disease the blood-lactic acid level in the rat shows a progressive rise and that this increase runs parallel with the increase in the number of trypanosomes (*T. evansi*) in the blood. Kligler, Geiger and Comaroff [1929], in a continuation of these investigations, concluded that the increased lactic acid production, as a result of the activities of the trypanosomes, results in an exhaustion of the alkali reserve [*cf.* also Geiger, Kligler and Comaroff, 1930].

These studies of the blood-sugar changes in trypanosomiasis indicate that some disturbance of the normal metabolism of sugar occurs in this disease. Most of the observations have been made, however, on small animals and in many instances with trypanosomes which differ markedly from those pathogenic to man, and it would be unwise to assume, without further evidence, that the results would be applicable to trypanosomiasis in man. Any disturbance of this type in the case of man would, however, be of clinical significance, although it would not necessarily contribute, directly or indirectly, to the decline of the host. No observations appear to have been made so far on the blood-sugar changes in human trypanosomiasis[1], although Sicé [1930] recorded a diminution in the C.S.F.-sugar in nervous trypanosomiasis, with a return, in the majority of cases, to more normal values when treatment was given.

In the present investigation, determinations of the resting blood-sugar values and the sugar tolerance for natives with trypanosomiasis and the values

[1] Since this work was completed and a preliminary report made (Annual Report for 1931 of the Institute for Human Trypanosomiasis Research, Entebbe, Uganda), a review has appeared in the April number for 1932 of the Tropical Diseases Bulletin [**29**, 285] of a paper by Walravens [*Ann. Soc. Belge de Med. Trop.* 1931, **11**, 213]. This author has studied the blood-sugar of 12 sleeping sickness patients before, during and after treatment and has observed no definite variations in the blood-sugar titre during these periods. In two of the patients, the titre was very low before treatment.

for normal natives have been made. The first study and the main part of this investigation was made on sleeping sickness patients kept in the hospital attached to the laboratory, and the controls were natives employed in the laboratory. The latter were selected in such manner that they might be regarded as normal in the sense that they were free from trypanosomiasis; probably every one had had malaria at some time and the majority had been infected with syphilis. The patients were given a liberal diet, the food being selected so that qualitatively and, as far as possible, quantitatively it resembled that of the controls. With the patients, and with practically all the controls, it was possible to be confident that no food had been taken in the morning before samples of blood were taken for fasting blood-sugar determinations. Repeated examinations were made on each patient and the effect of treatment was noted. Twelve patients were thus examined over varying lengths of time, the number available being limited partly by the accommodation but mainly by the disinclination on the part of the native to stay in hospital. With one notable exception, it was extremely difficult to persuade the patients to stay for longer than one month under observation, and as soon as an improvement in their general condition occurred as a result of treatment, they expressed a very strong desire to return to their homes. The results of these laboratory investigations, in spite of the rather small number of patients examined, are considered of value as a more or less systematic study of these selected cases of human trypanosomiasis. The original programme included determinations of the resting blood-sugar of sleeping sickness patients and normals in the West Nile area of the Uganda Protectorate, where it was hoped that a large number of treated and untreated cases might be studied. Owing to circumstances over which the author had no control, however, it was not possible to carry out the second half of this programme.

All the blood-sugar determinations were made by the Hagedorn and Jensen method [1923] [*cf.* Beaumont and Dodds, 1930], this method being selected as the most suitable method for work in the field. A few drops of capillary blood were collected, early in the morning, from a finger of the fasting patient (or control), no food having been taken by the subject since the previous evening. This blood was used immediately for sugar determinations. Duplicate determinations were made with each sample, and the figures given here represent the mean of the two values obtained.

Experimental.

Sleeping sickness patients were selected by the Medical Officers in various parts of Uganda and were sent to Entebbe[1]. These patients were kept in the small hospital attached to the Human Trypanosomiasis Institute at Entebbe,

[1] The author would like to take this opportunity of thanking Major G. J. Keane, Director of Medical and Sanitary Services, the Uganda Protectorate, and Dr S. W. T. Lee for their generous help in this and other matters. In particular, he is indebted to them for the details of the previous history of these patients and for the very satisfactory arrangements made for him by the Medical Department when the author went on safari to the West Nile district in 1931.

and samples of blood were taken from each patient every day (except Sundays) and examined for trypanosomes, malarial parasites, *etc.* Repeated examinations were also made by the red cell adhesion test [Duke and Wallace, 1930; Wallace and Wormall, 1931] and, on occasions, the C.S.F. was collected and examined. In other cases, the blood of the patient was injected into monkeys, guinea-pigs or mice and further examination of the trypanosomes made. The assumption was made that in all these cases the infection was due to *T. gambiense*, since *T. rhodesiense* infections in man appear to be of very infrequent occurrence in Uganda (*cf.*, however, the report of a case of *T. rhodesiense* infection in man in Uganda by Duke [1930]). The results of the red cell adhesion test, the behaviour of the trypanosome in monkeys and other animals (*i.e.* duration of infection and the result of drug treatment) and the symptoms of the patient were, with one possible exception, in entire agreement with the views that the trypanosome concerned was *T. gambiense*. The possible exception was the infection in the patient Kibuka; this matter will be discussed more fully in a subsequent paper [Wormall, 1932].

The patients had a mixed diet which was particularly rich in carbohydrate, the main bulk of the food being taken in the form of sweet potatoes, muhogo flour and sugar cane. Fish was given on most days and meat twice a week. The daily ration included also small quantities of milk, tea and salt. With most of these patients, drug treatment was started after the patient had been at Entebbe for about three or four weeks, the dates of the injections being given in Tables I and II. Treatment during the blood-sugar investigations consisted of the intravenous injection of a solution containing 1 g. of Bayer 205, except in one case where tryparsamide was used. Regular injections of tryparsamide and Bayer 205 were subsequently given to all the patients. At one period during this study, one of the patients developed malaria and was treated with quinine. No blood-sugar determinations were made on this patient during this period or on the seven days following quinine administration.

The patients can be divided into three groups, although it is not suggested that there should be any sharp differentiation between these groups:

(*a*) *Those with little or no previous drug treatment*:

Sumba—no treatment.

Lisuwa, Manika, Oditi and Otenyi (with one injection of tryparsamide each).

Kibuka (with one injection of tryparsamide 2 years previously).

(*b*) *Those with a moderate amount of drug treatment*:

Orachi, Osanda and Atoyo.

(*c*) *Old-standing cases* (*with previous drug treatment*):

Wanjira, Zenesio and Achan.

Age, sex and previous history of these patients.

Sumba. Male, age 25–30 years. Trypanosomes first detected (in the blood) 24. xi. 30. No treatment given prior to arrival at Entebbe, 8. xii. 30.

Lisuwa. Male, age 40. Trypanosomes first detected in Feb. 1931 (gland juice). Received 2·5 g. tryparsamide on 18. ii. 31. Arrived at Entebbe, 28. ii. 31.

Manika. Male, age 20. Trypanosomes first detected in gland juice in Sept. 1930. Received 2·5 g. tryparsamide on 15. ix. 30. Arrived at Entebbe, 27. ix. 30.

Oditi. Male, age 19. Trypanosomes first detected in the gland juice in Feb. 1931. Received 2·5 g. tryparsamide on 18. ii. 31. Arrived at Entebbe, 28. ii. 31.

Otenyi. Male, age 26. Trypanosomes first detected in the gland juice early in Dec. 1930. Received 2·5 g. tryparsamide on 5. xii. 30. Arrived at Entebbe on 21. xii. 30.

Kibuka. Male, age 25–30. Trypanosomiasis diagnosed in 1928, but no further official details were available. (Patient stated that he received one injection only in 1928; none in 1929 or 1930.) Trypanosomes detected in the blood and gland juice on 4. iii. 31. Arrived at Entebbe, 8. iii. 31.

Orachi. Male, age 29. Trypanosomes first detected in the gland juice. Received injections of tryparsamide (2·5 g. each time) on 7. xi. 30, 14. xi. 30, 2. xii. 30 and 9. xii. 30. Arrived at Entebbe, 21. xii. 30.

Osanda. Male, age 10. Trypanosomes first detected in the gland juice 1930. Received 20 injections of tryparsamide (total of 55 g.) from 18. ii. 30 to 15. ix. 30; over practically the whole of this period gland punctures revealed the presence of trypanosomes. This infection appeared, therefore, to be resistant to treatment with tryparsamide, and experiments carried out by Dr J. M. Wallace showed that guinea-pigs infected with this trypanosome were not completely cured by the normal sterilising doses of atoxyl. A large number of adhesion tests were carried out with the serum of this patient and others with the trypanosomes of animals inoculated with his blood, and in all these tests the infection appeared to be a typical *gambiense* infection.

Atoyo. Male, age 8. Trypanosomes first detected in the gland juice in June, 1929. Received 8·5 g. tryparsamide and 1 g. Bayer 205 from 21. i. 31 to 9. iv. 31. Arrived at Entebbe, 22. iv. 31.

Wanjira. Male, age 35. Trypanosomiasis diagnosed in 1926. Received 14 g. tryparsamide and 4 g. Bayer 205 in 1927, 42 g. tryparsamide in 1928, and 6 g. tryparsamide in 1929. In 1930, he received 12 g. tryparsamide during Sept., Oct. and Nov. Sent to Entebbe as a possible case of relapse, 8. xii. 30.

Zenesio. Male. Early history not available. 14 g. tryparsamide and 4 g. Bayer 205 given from May to Sept. 1930. Trypanosomes present in the blood on 12. v. 30 and on several subsequent occasions. Arrived at Entebbe in May, 1930.

Achan. Female, age 40. Trypanosomes detected in gland juice in Aug. 1929. From Aug. to Nov. 1929, she received 35 g. tryparsamide, and from 26. viii. 30 to 9. iv. 31 she received 32 g. tryparsamide and 1·5 g. Bayer 205. Arrived at Entebbe, 22. iv. 31.

Wanjira and Sumba were sent from Tororo (East Province, Uganda); Atoyo and Achan came from Gulu, North Province; all the other patients came from the West Nile district of the Uganda Protectorate.

The results of the fasting blood-sugar determinations with the sleeping sickness patients are given in Tables I and II. The date of each examination and the results of the examination of blood films for trypanosomes are also recorded. Whenever drug treatment (the intravenous injection of a solution of Bayer 205 or tryparsamide) was given, reference is made to it in the Tables in order that a direct study might be made of the influence of this treatment on the fasting blood-sugar level. For convenience the results with patients of group (*a*) are given in Table I and those for groups (*b*) and (*c*) in Table II, but it is not suggested that this division into groups is very clear cut, or that it has any special significance. Table III gives average fasting blood-sugar values for these patients.

In these blood-sugar investigations it was necessary to have, for purposes of comparison, satisfactory values for the fasting blood-sugar of natives of Uganda, and in Table IV the results of such determinations with 12 "normal" natives (four determinations with each native, made at approximately weekly intervals) are given. These control natives were normal in the sense that they did not suffer from trypanosomiasis; all of them were apparently healthy, and

Table I. *Fasting blood-sugar values with trypanosomiasis patients in group (a).*

Sugar values as mg. per 100 cc.

Date	Blood-sugar	Blood examination*	Date	Blood-sugar	Blood examination	Date	Blood-sugar	Blood examination
	Sumba			Lisuwa			Manika	
16. xii. 30	82	+	5. iii. 31	106	+	29. ix. 30	95	–
17. xii. 30	97	+	10. iii. 31	98	+	3. x. 30	98	–
19. xii. 30	82	+	13. iii. 31	102	+	10. x. 30	97	–
22. xii. 30	82	+	18. iii. 31	91	+	13. x. 30	88	–
24. xii. 30	94	+	24. iii. 31	91	+	16. x. 30	99	–
29. xii. 30	89	+	26. iii. 31	98	+	20. x. 30	99	–
3. i. 31	72	+	31. iii. 31	99	+	23. x. 30	1 g. Bayer 205	
9. i. 31	83	+	8. iv. 31	94	–	24. x. 30	100	–
16. i. 31	1 g. Bayer 205		9. iv. 31	1 g. Bayer 205		30. x. 30	88	–
20. i. 31	90	–	15. iv. 31	99	–	31. x. 30	1 g. Bayer 205	
24. i. 31	100	–				1. xi. 30	95	–
29. i. 31	93	–				6. xi. 30	97	–
6. ii. 31	95	–						
	Oditi			Otenyi			Kibuka	
5. iii. 31	105	+	22. xii. 30	69	–	10. iii. 31	93	–
10. iii. 31	96	+	24. xii. 30	86	–	13. iii. 31	100	–
13. iii. 31	100	+	29. xii. 30	93	+	18. iii. 31	90	–
18. iii. 31	87	+	3. i. 31	96	+	20. iii. 31	1 g. Bayer 205	
24. iii. 31	77	–	7. i. 31	1 g. Bayer 205		24. iii. 31	60	–
24. iii. 31	1 g. Bayer 205		9. i. 31	96	–	26. iii. 31	81	–
31. iii. 31	91	–				31. iii. 31	87	–
8. iv. 31	84	–				8. iv. 31	77	–
9. iv. 31	1 g. Bayer 205					9. iv. 31	1 g. Bayer 205	
15. iv. 31	72	–				15. iv. 31	94	–

* Examination of wet and stained blood films for trypanosomes.

Table II. *Fasting blood-sugar values with trypanosomiasis patients of groups (b) and (c).*

Sugar values as mg. per 100 cc.

Date	Blood-sugar	Blood examination	Date	Blood-sugar	Blood examination	Date	Blood-sugar	Blood examination
	Osanda			Wanjira			Zenesio	
6. x. 30	77	+	16. xii. 30	99	–	29. ix. 30	57	–
8. x. 30	91	+	17. xii. 30	105	–	6. x. 30	69	–
10. x. 30	93	+	19. xii. 30	98	–	10. x. 30	70	–
13. x. 30	99	+	22. xii. 30	91	–	11. x. 30	1 g. Bayer 205	
16. x. 30	90	–	24. xii. 30	97	–	13. x. 30	107	–
20. x. 30	93	+	29. xii. 30	100	–	16. x. 30	86	–
24. x. 30	82	–	3. i. 31	96	–	20. x. 30	77	–
25. x. 30	1 g. tryparsamide		9. i. 31	98	–	24. x. 30	79	–
30. x. 30	79	+	20. i. 31	101	–	30. x. 30	73	–
30. x. 30	1 g. Bayer 205		24. i. 31	101	–	1. xi. 30	85	–
1. xi. 30	99	–	29. i. 31	104	–	6. xi. 30	83	–
6. xi. 30	94	–	6. ii. 31	107	–	14. xi. 30	72	–
8. xi. 30	1 g. Bayer 205					25. xi. 30	74	–
14. xi. 30	91	–				27. xi. 30	86	–
21. xi. 30	1 g. Bayer 205							
25. xi. 30	87							
	Orachi			Atoyo			Achan	
22. xii. 30	85	+	29. iv. 31	91	–	6. v. 31	79	–
24. xii. 30	90	+	6. v. 31	86	–	12. v. 31	77	–
29. xii. 30	86	+	12. v. 31	82	–			
3. i. 31	87	–						
7. i. 31	1 g. Bayer 205							
9. i. 31	78	–						

Table III. *Average values for fasting blood-sugar of trypanosomiasis patients.*

Blood-sugar as mg. per 100 cc.

Patient	Before treatment at Entebbe		Before and after treatment (all values)	
	No. of determinations	Average	No. of determinations	Average
Sumba	8	85	12	88
Lisuwa	8	97	9	98
Manika	6	96	10	96
Oditi	5	93	8	89
Oteryi	4	86	5	88
Kibuka	3	94	8	85
Osanda	7	89	12	90
Wanjira	12	100	12	100
Zenesio	3	65	13	78
Orachi	4	87	5	85
Atoyo	3	86	3	86
Achan	2	78	2	78

Table IV. *Fasting blood-sugar (mg. per 100 cc.) normals.*

	Determinations				
	1	2	3	4	Average
Lolenti	86	89	81	84	85
Joseph	107	93	95	98	98
Mikaili Mayanja	83	82	88	90	86
Butulumayo	88	94	85	85	88
Matayo Kiwanuka	92	85	102	99	94
Yusufu Kolokojo	99	94	96	100	97
Abinayo Kitakule	104	100	99	109	103
Yokana	97	101	98	92	97
Alajabu	89	92	93	88	90
Asiri Mutasa	77	95	75	82	82
Erika Kasozi	98	81	85	88	88
Isaka Serwangu	94	104	82	96	94

Table V. *Carbohydrate tolerance tests.*

Blood-sugar values (mg. per 100 cc.) after taking 50 g. glucose.

	Before glucose	½ hr. after glucose	1 hr.	1½ hrs.	2 hrs.	3 hrs.
Manika	98	111	166	141	107	100
Osanda 1)	91	137	131	124	108	91
,, (2)	90	104	100	102	88	77
,, (3)	93	125	121	90	70	91
Zenesio (1)	70	107	117	105	105	77
,, (2)	72	97	148	109	105	74
,, (3)	74	124	162	122	115	87
Sumba (1)	97	102	127	119	112	94
,, (2)	94	104	127	135	130	105
Lisuwa (1)	94	123	152	114	89	87
,, (2)	99	153	166	97	92	93
Normal native (1)	84	109	101	100	88	77
,, (2)	86	161	201	181	115	90
,, (3)	81	123	111	106	74	82
,, (4)	91	125	121	110	89	90
,, (5)	92	131	137	129	92	85

were not suffering from malaria during the period when their blood-sugar values were determined.

In view of the suggestion made by several authors that in trypanosomiasis of small animals there is a general disturbance of carbohydrate metabolism involving a derangement of the storage of glycogen by the liver, it was decided to make glucose tolerance tests with certain of the sleeping sickness patients and normal natives. Table V gives the results of the tests with the patients and a few typical results with controls. None of the patients and only one of the controls (normal native No. 2 in the Table) passed sugar in the urine during these tolerance tests.

Discussion.

The results of determinations of the fasting blood-sugar of natives suffering from trypanosomiasis (Tables I and II) indicate that in this condition the fasting blood-sugar is not markedly lower than that for a native who does not suffer from this disease (*cf.* Table IV for the controls). Marked variations from time to time in the fasting blood-sugar of any one patient certainly occur, but such variations also occur with normal natives. The fluctuations are perhaps somewhat greater with the patients, but the difference between the patients and the controls in this respect does not appear to be significant. Nor are the average values for the sleeping sickness patients very definitely lower than the average values for the control normal natives (*cf.* Tables III and IV), and it is only in isolated instances that there is any suggestion of a marked hypoglycaemia.

It is perhaps difficult to decide what might be regarded as the lower limit for normal fasting blood-sugar values. With the controls used in this work (natives of various tribes in Uganda) the lowest value was 75 mg. per 100 cc. In some investigations carried out on two tribes in Kenya (Special Report Series, No. 155, of the Medical Research Council, Edited by J. B. Orr and J. L. Gilks, 1931), figures of 77 to 136 mg. per 100 cc. are given for the fasting blood-sugar values of normal natives, with perhaps slightly lower values in cases of tropical ulcer (one low value of 65 mg.). In the course of another investigation, Dr F. W. Vint and Dr D. Harvey in Nairobi, using a modification of the Hagedorn and Jensen blood-sugar method, obtained, with natives, fasting blood-sugar values which were usually about 100 mg., or slightly more, per 100 cc. The lowest value in 25 determinations was 75 mg. per 100 cc. (private communication from Dr Harvey in 1931). From these figures for normal natives in Uganda and Kenya, it might be considered, therefore, that the following values for the sleeping sickness patients investigated in the work described here were subnormal; 57, 69, 70, 72 and 73 mg. per 100 cc. with Zenesio, 60 mg. with Kibuka and 69 mg. with Otenyi. These low values, although they may offer some support for the view that there is a hypoglycaemia in human trypanosomiasis, do not occur sufficiently often to suggest a very marked disturbance of carbohydrate metabolism in this disease. The results of the glucose tolerance tests with the patients and a few

of the controls (Table V) also indicate that, in the early stages of trypanosomiasis at least, there is no very serious inability of the liver to deal with glucose. Several of the sugar tolerance curves were of the lag type, and this finding is in agreement with the observations of Bhatia and Coelho [1925], who carried out tests on Indians. The latter authors obtained values between 83 and 246 mg. per 100 cc. for the fasting blood-sugar of these people accustomed to a vegetarian diet.

On theoretical grounds alone it is perhaps rather difficult to conceive of a hypoglycaemia in man due entirely to a heavy sugar consumption by the trypanosomes. With a supply to the body of 300 g. at the minimum and usually considerably more than 400 g. of carbohydrate daily in the form of food, it appears unlikely that the amount of sugar used by the trypanosomes would, in itself, result in any marked diminution in the fasting blood-sugar level or in the amount of glycogen stored in the liver and muscles of the patient. It may be, however, as has been suggested by several authors as a result of experiments on smaller animals, that the consumption of glucose by the trypanosomes leads indirectly (perhaps as a result of the production of certain metabolites) to a disturbance of the mechanism concerned with maintaining a minimum blood-sugar level, and it is perhaps only to be expected that these disturbances would not be manifested until the infection had been established for some time. For this reason it would be of interest to know whether there are any marked changes (diminution in glucose content, *etc.*) in the C.S.F. of patients in the advanced stages of sleeping sickness.

Intimately linked up with this problem of carbohydrate metabolism is the oxygen consumption of the trypanosomes, and it is not impossible that a serious disturbance of normal metabolic processes occurs as a result of the trypanosomes depriving certain organs and tissues of part of their supplies of carbohydrate and oxygen. In the course of this investigation in Uganda it was noticed that the blood taken from the finger of the trypanosomiasis patient was in practically every case considerably darker than samples of blood from the control natives. This difference was best observed when the blood was collected, with the minimum of shaking, in a very small conical test-glass containing a trace of potassium oxalate, or preferably when the blood was immediately sucked up under a layer of liquid paraffin into a capillary pipette containing potassium oxalate or sodium citrate, and observed against a white background. The darker, less oxygenated, bloods could then be picked out at once. Similar differences were observed in tests with samples of blood from monkeys infected with trypanosomes and normal monkeys. This darkness did not appear to be evident only when trypanosomes were present in the blood, since it was observed with these sleeping sickness patients when no trypanosomes could be detected in wet and stained blood films. Thus it does not appear to be due entirely to the using up for metabolic purposes of part of the oxygen of the oxyhaemoglobin by trypanosomes present in the sample of blood tested. If these samples were then shaken with air they became as red as the control

samples similarly shaken, but when they had stood again for a short time there was usually but not invariably more reduction in the blood from the sleeping sickness patients. With the blood of infected monkeys, and to a lesser extent with the human bloods which contained trypanosomes, this later reduction can undoubtedly be attributed to a diminution of the oxygen content as a result of the oxidation of glucose by the trypanosomes [*cf.* Yorke, Adams and Murgatroyd, 1929, p. 510]. In the instances where no significant number of trypanosomes are present in the blood, these observations seem to indicate a higher reducing power of the plasma. It was intended to carry out experiments along these lines, including determinations of the oxygen content and capacity of arterial and venous bloods of the sleeping sickness patients and the reducing power of the serum or plasma, but unfortunately it was not possible to complete these investigations.

Summary.

1. In view of the findings of various authors, that in trypanosomiasis of small animals there is a significant hypoglycaemia, which according to some is partially, if not entirely, responsible for the decline of the host, repeated blood-sugar determinations have been carried out on 12 sleeping sickness patients in Uganda (*T. gambiense* infections).

2. Determinations carried out with these patients and with suitable controls (natives not suffering from trypanosomiasis) over varying periods of time indicate that in man there is no general hypoglycaemia during the earlier stages of this disease. In certain instances, however, a definitely low blood-sugar value was obtained, these low values being significantly lower than the lowest value with a normal native.

3. Treatment of these patients with Bayer 205 gives variable results in relation to the blood-sugar; sometimes there is a fall, sometimes a rise, and in other cases no change in the blood-sugar level. With the patient with the lowest blood-sugar values, the injection of 1 g. Bayer 205 resulted in a speedy return to normal (or perhaps slightly high) values.

4. No relationship could be demonstrated between the blood-sugar level and the presence in or absence from the blood of trypanosomes.

5. Glucose tolerance tests carried out on a few of the patients and some of the controls do not suggest that in trypanosomiasis there is a very marked impairment of the capacity of the liver to deal with glucose.

The author has pleasure in acknowledging the help which he received at various times during the course of this investigation from Dr H. L. Duke, Director of the Human Trypanosomiasis Institute, and from Drs A. R. D. Adams and J. M. Wallace.

REFERENCES.

Beaumont and Dodds (1930). Recent advances in medicine, 5th edition. (J. and A. Churchill, London.)

Bhatia and Coelho (1925). *Ind. J. Med. Res.* **13**, 41.

Bruynoghe, Dubois and Bouckaert (1927). *Bull. Acad. Roy. Méd. Belge*, **7**, 142.

Cordier (1927). *Compt. Rend. Soc. Biol.* **96**, 971.

Dubois (1928). *Compt. Rend. Soc. Biol.* **99**, 656.

—— and Bouckaert (1927). *Compt. Rend. Soc. Biol.* **96**, 431.

Duke (1930). *Trans. Soc. Trop. Med. Hyg.* **24**, 201.

—— and Wallace (1930). *Parasitology*, **22**, 414.

Fenyvessy (1926). *Biochem. Z.* **173**, 289.

—— and Scheff (1930). *Biochem. Z.* **221**, 206.

Geiger, Kligler and Comaroff (1930). *Ann. Trop. Med. Parasit.* **24**, 319. Cited from *Trop. Dis. Bull.* 1931, **28**, 374.

Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.

Kligler and Geiger (1928). *Proc. Soc. Exp. Biol. Med.* **26**, 229.

—— —— and Comaroff (1929). *Ann. Trop. Med. Parasit.* **23**, 325. Cited from *Trop. Dis. Bull.* 1930, **27**, 241.

Knowles and Das Gupta (1928). *Ind. J. Med. Res.* **15**, 997.

Linton (1929). *Ann. Trop. Med. Parasit.* **23**, 307.

—— (1930). *J. Exp. Med.* **52**, 103 and 695.

Regendanz (1929). *Arch. Schiffs. Tropen. Hyg.* **33**, 242.

—— and Tropp (1927). *Arch. Schiffs. Tropen. Hyg.* **31**, 376.

Scheff (1928). *Biochem. Z.* **200**, 309.

—— (1929). *Magyar Orvosi Arch.* **30**, 110 and 120. *Chem. Abst.* 1929, 3508.

Schern (1925). *Zentr. Bakt.* Orig. **96**, 356, 360, 362, 440, 444 and 451.

—— (1927). *Deutsch. med. Woch.* **53**, 106.

—— (1928). *Biochem. Z.* **193**, 264.

Sicé (1930). *Bull. Soc. Path. Exot.* **23**, 640. Cited from *Trop. Dis. Bull.* 1930, **27**, 823.

Wallace and Wormall (1931). *Parasitology*, **23**, 346.

Wormall (1932). *Parasitology* (in the Press).

Yorke, Adams and Murgatroyd (1929). *Ann. Trop. Med. Parasit.* **23**, 501.

Zotta and Radacovici (1929). *Arch. Roumaines Path. Exp. Microbiol.* **2**, 55 and *Compt. Rend. Soc. Biol.* **102**, 129.

CCXI. A METHOD OF DETERMINATION OF SOME BIOLOGICALLY IMPORTANT ALDEHYDES AND KETONES, WITH SPECIAL REFERENCE TO PYRUVIC ACID AND METHYLGLYOXAL.

By FRANCIS PAUL CLIFT[1] and ROBERT PERCIVAL COOK[2].

From the Biochemical Laboratory, Cambridge.

(*Received September 11th*, 1932.)

I. Introduction.

The importance of various types of unsaturation in molecules in intermediary metabolism is a subject to which attention is being increasingly devoted. During the study of some of the intermediary mechanisms of carbohydrate metabolism in the liver we have been interested in one type of unsaturated linkage, namely that of the carbonyl group. Aldehydes and ketones possess, as is well known, the capacity of forming hydrazones and osazones, which capacity is sometimes used in their identification and estimation. Another property is their power of forming compounds with bisulphites. This property has long been used as a method of estimating acetaldehyde [Ripper, 1900]. That this method can be used for other aldehydes and ketones was shown by Cook [1930] who based on it a method for the estimation of pyruvic acid.

We have extended the method to other aldehydes and ketones with, we feel, some measure of success and can utilise the method, for example, for the determination of a mixture of pyruvic acid with acetaldehyde or methylglyoxal in a single sample. The method shares the disadvantage of unspecificity with the other methods of estimation of carbonyl compounds, there being inherently no specific method of estimation for a mixture of two carbonyl compounds except in so far as the hydrazones or osazones of acids may be separated from those of non-acidic compounds by their solubility in sodium carbonate [Dakin and Dudley, 1913].

Owing to the considerable attention focused on the position of methylglyoxal in intermediary metabolism numerous methods have been proposed for its estimation. As will be shown later the question of the estimation of methylglyoxal in tissues is rather a difficult one.

Wendel [1932] has given a very complete account of the methods that have been used for the estimation of pyruvic acid. In addition to pure pyruvic acid we have devoted some attention to the subject of the polymerides of this acid.

[1] Reginald John Smith Research Student.

[2] Beit Memorial Research Fellow.

We feel that our method offers a great advantage in the rapidity with which estimations can be carried out with the minimum of apparatus and the necessity of only one standard solution.

II. Principle and experimental details of estimations of a single substance.

In the presence of excess of sodium bisulphite the aldehydes and ketones studied react at room temperature to form bisulphite compounds. The principle of the method is to remove the excess bisulphite from the solution by adding the required amount of iodine. The bisulphite compounds are then hydrolysed by saturating the solution with sodium bicarbonate, and the liberated bisulphite is titrated with a dilute standard iodine solution. Thus in general 1 cc. of *N* iodine is equivalent to half the molecular weight of the aldehyde or ketone expressed in mg. The principle is similar to that described by Clausen [1922] and amplified by Friedemann, Cotonio and Shaffer [1927].

The conditions which we have found most suitable are as follows. A 5 cc. aliquot of the solution to be estimated is added to 1·0 cc. of approximately *M* sodium bisulphite solution. The solution is allowed to stand for at least 15 minutes and then a few drops of starch solution are added. The excess bisulphite is then removed by running in approximately *N*/10 iodine solution (conveniently from a burette) until the blue colour indicates the presence of a slight excess of iodine. The blue colour is discharged by the addition of a few drops of approximately *N*/100 sodium thiosulphate solution. The end-point is then adjusted to a faint blue colour by means of the dilute standard iodine solution (*N*/100, *N*/200 or *N*/500 according to the concentration of the carbonyl compound). 5 cc. of an aqueous suspension of sodium bicarbonate are then added and the standard iodine again run in until the blue colour of the indicator is of the same intensity as it was before the addition of the bicarbonate.

It is important that the standard iodine should be freshly diluted with oxygen-free water and also that the bicarbonate suspension should be free from oxygen. The latter end is readily achieved by blowing a stream of carbon dioxide through it for a few minutes immediately before use.

In most cases the reaction is stoichiometric, but where this is not the case an empirical relationship has been found as described below.

With most of the substances studied the reaction between the carbonyl compound and the sodium bisulphite is complete within a very few minutes. With certain substances (notably acetaldehyde and methylglyoxal) it is necessary to allow the solution to stand for 15 minutes.

It is also important when dealing with solutions of biological origin that the reaction should be slightly acid before adding the bisulphite. (See note in section VIII on the effect of glucose on the titration.) The acidity should not, however, be greater than *N*/10, as low results are obtained in strongly acid solution.

With methylglyoxal the acidity of the 5 cc. aliquot should not be greater than *N*/50. The acid most likely to be present in solutions of biological origin is trichloroacetic acid. Table I shows the effect of various concentrations of this acid on the estimation of methylglyoxal.

Table I.

Mg. methylglyoxal		Normality of trichloroacetic acid	% of theory
Added	Found		
1·16	1·07	0·122	92
1·16	1·12	0·061	97
1·16	1·16	0·024	100
0·436	0·419	0·122	96
0·436	0·436	0·024	100

III. Principle and experimental details of the estimation of a mixture of an alkali-stable and an alkali-labile substance.

While certain of the biologically important carbonyl compounds are both non-volatile and stable towards hot dilute alkali, the majority disappear nearly completely when the solution is heated to 100° with dilute alkali in an unstoppered flask. We have found it possible to make use of this difference in properties to effect an approximate estimation of the components of a mixture of such an alkali-stable compound with one which is destroyed by alkali.

With the alkali-stable compound which we have investigated, namely pyruvic acid, the percentage remaining after treatment with alkali under the conditions described below is 93 %, while with the alkali-labile compounds (methylglyoxal, acetaldehyde, glyceraldehyde, dihydroxyacetone *etc.*) a few per cent. only remain. The method is to titrate an aliquot in the usual manner and to titrate another aliquot after heating with alkali.

It is found that the fraction remaining undestroyed is independent of the initial concentration in the case of the stable compound, and approximately a rectangular hyperbolic function of it in the case of the labile compounds. Thus if y mg. of the labile compound are present, and f is the fraction remaining after treatment with alkali,

$$yf = K.$$

The constant K has been determined for a number of compounds (see section V).

Let F = fraction of the stable substance remaining after treatment with alkali,

x = mg. of the stable substance originally present,

1 cc. $N/10$ I = p mg. alkali-labile substance,

1 cc. = q mg. alkali-stable substance.

Then if the titrations before and after the alkali treatment are T_1 and T_2 cc. respectively,

$$x = \frac{q}{F}\left(T_2 - \frac{K}{p}\right)$$

$$y = p\left(T_1 - \frac{T_2}{F}\right) + \frac{K}{F}.$$

The conditions we have chosen for the treatment with alkali are as follows. A 5 cc. aliquot of the solution is placed in a 125 cc. Erlenmeyer flask together with a drop of thymolphthalein solution. 3 cc. of N NaOH are then added in excess of the amount required just to cause the solution to turn blue. The flask is then placed for 1 hour on the boiling water-bath. At the end of this time the contents of the flask are rendered slightly acid with N H_2SO_4. The exact concentration of alkali and the exact time of heating are not of great importance, at least in the case of methylglyoxal. Table II shows the effect of varying these factors.

Table II.

5 cc. aliquots of methyglyoxal, each containing 0·30 mg.

cc. N NaOH	Time of heating (hours)	Fraction remaining
0·5	1·0	0·045
1·0	1·0	0·030
2·0	1·0	0·027
4·0	1·0	0·030
3·0	0·5	0·033
3·0	1·0	0·034
3·0	1·5	0·034
3·0	2·0	0·033

IV. Examples of the estimation of a single substance.

(1) *Pyruvic acid.*

Pyruvic acid (B.D.H.) was repeatedly distilled *in vacuo* to remove the α-keto-γ-valerolactone-γ-carboxylic acid which may constitute up to 30 % of the average commercial sample of pyruvic acid. The acid was frozen out and the crystals, M.P. 10° (approx.), dissolved in water. No attempt was made to obtain this very deliquescent substance in an anhydrous condition, and the stock solutions were standardised by the method of Wieland [1923]. The results obtained by this method agreed perfectly with those obtained by titration with alkali.

Table III shows the results of bisulphite titrations of the stock solutions.

Table III.

1 cc. *N*/10 I≡4·4 mg. pyruvic acid.

Mg. pyruvic acid		
Used	Found	% yield
22·0	22·0	100
5·58	5·49	98
2·79	2·88	103
2·20	2·20	100
1·39	1·35	97
0·697	0·700	100
0·349	0·351	101
0·221	0·225	102

Note on certain degradation products of pyruvic acid.

(*a*) *α-Keto-γ-valerolactone-γ-carboxylic acid.* As first observed by Wolff [1899] pyruvic acid undergoes spontaneous condensation to this substance. As indicated above commercial samples of pyruvic acid may contain up to 30 % of the lactone. Other decomposition products are also present, as indicated by the odour and colour of old samples of the acid. In order to determine the behaviour of these impurities in the method of estimation we have studied the residue which remains after distillation of pyruvic acid *in vacuo*. This syrupy residue crystallises after standing for a few hours *in vacuo* over quicklime [Wolff, 1901], and consists largely of α-keto-γ-valerolactone-γ-carboxylic acid. A solution of the dry substance takes up iodine in the presence of sodium bicarbonate. The end-point is ill-defined, but the amount of standard iodine taken up corresponds to about half that consumed by an equal weight of pyruvic acid. The titre is not increased by previously allowing the solution to stand with sodium bisulphite.

We have observed that dilute solutions of commercial pyruvic acid give a higher bisulphite titration after long standing than when freshly made up. This is probably due to hydrolysis of the lactone to pyruvic acid. Thus a solution with an initial titre corresponding to 28·0 mg./cc. of pyruvic acid gave after standing for 4 months at about 4° in the presence of toluene a titre corresponding to 33·1 mg./cc. Thus it appears that a convenient way of storing pure pyruvic acid is as the aqueous solution of the free acid.

(*b*) *The so-called* "para*pyruvate.*" The so-called *para*pyruvates are formed when neutral or slightly alkaline aqueous solutions of pyruvates are boiled or allowed to stand for long periods at room temperature. We would like to point out that statements have been made that a substance which, on alkaline hydrolysis, reverts to pyruvic acid is formed when a neutral pyruvate solution is incubated for long periods with bacteria [see Quastel and Wooldridge, 1929, p. 133]. The observations of Wolff [1899] and de Jong [1901; 1902] indicate that the so-called *meta-* and *para*pyruvates are formed under the same conditions without the bacteria.

Barium *para*pyruvate was prepared by allowing a saturated solution of crystalline barium pyruvate to stand for several days at room temperature [de Jong, 1902]. The amorphous white solid which separated was washed thoroughly by repeated shaking with water and air-dried to constant weight. The composition of the product thus obtained is, according to de Jong, $C_6H_6O_6Ba, 4H_2O$. The barium was removed by shaking an accurately weighed amount with a slight excess of dilute sulphuric acid, filtering off the barium sulphate, and making up the filtrate and washings to a known volume with water.

The *para*pyruvic acid thus prepared was found to have a capacity of uniting with bisulphite of approximately one-third that of the equivalent amount of pyruvic acid. On this basis, that 1 cc. $N/10$ I$\equiv$13·2 mg. of *para*pyruvic acid[1], the figures given in Table IV were obtained.

Table IV.

mg. *para*pyruvic acid*	
Used	Found
15·3	16·5
7·6	8·2
3·8	3·8
1·9	1·8
0·95	0·80

* Expressed as pyruvic acid.

When aliquots of the solution were heated with alkali as described in section III a strong yellow colour developed. It was not possible to reach a sharp end-point in a bisulphite titration after this treatment. However, the approximate titrations obtained were uniformly higher than before the alkali-treatment.

(2) *Methylglyoxal.*

Methylglyoxal was prepared by the method of Fischer and Taube [1924], immediately redistilled *in vacuo* and weighed by transferring it to a tared glass-stoppered flask of water as soon as it melted. The solutions thus obtained were perfectly clear. The empirical relationship between the amount of iodine used in a bisulphite titration and the weight of methylglyoxal present was perfectly consistent with different preparations. We have, however, been unable to reproduce quantitatively the results obtained by Kuhn and Heckscher [1926] who describe an empirical method of estimating methylglyoxal by oxidation with iodine in a sodium bicarbonate-carbonate buffer mixture. There seemed to us to be an ambiguity in the phrase "eines Gemisches von 0·2 molarer Na_2CO_3- und 0·2 molarer $NaHCO_3$-Lösung." We therefore carried out estimations on our stock solutions according to their method, using (*a*) a mixture containing 0·2 mols. Na_2CO_3 and 0·2 mols. $NaHCO_3$ in one litre, and (*b*) the above mixture diluted to twice its volume with water.

Estimations carried out on a solution containing 9·875 mg. of methylglyoxal yielded the following results:

Mixture (*a*) 13·20, 13·23, 13·23 mg.
,, (*b*) 12·11, 11·75, 11·78 mg.

When methylglyoxal is titrated according to our method, the iodine titre does not correspond to a stoichiometric union of either one or two molecules of bisulphite with one molecule of methylglyoxal, but to an intermediate

[1] Expressed as pyruvic acid.

value. For quantities between 0·2 and 2·0 mg. of methylglyoxal (in 5 cc.) a linear relationship holds between the titre and the amount of methylglyoxal, 1 cc. *N*/10 I being equivalent to 2·85 mg. of methyglyoxal. Over this range the end-points are perfectly sharp, but with greater concentrations of methylglyoxal this is not the case, and the magnitude of the titre indicates the union of more bisulphite with the methylglyoxal.

The results of some estimations are shown in Table V.

Table V.

1 cc. *N*/10 I≡2·85 mg. methylglyoxal.

Mg. methylglyoxal		
Used	Found	% yield
1·99	1·91	96
1·44	1·45	101
0·996	1·01	101
0·718	0·738	103
0·498	0·491	99
0·249	0·251	101

This relationship being an empirical one, it is important that the conditions which we have described should be strictly observed. The volume of the solution of methylglyoxal is 5·0 cc., and the sodium bisulphite is added as 1·0 cc. of *M* solution. The above relationship does not hold when the concentration of bisulphite differs greatly from that specified.

It is also important that the normality of the methylglyoxal solution with respect to acid should not be greater than *N*/50 (Table I).

(3) *Dihydroxyacetone.*

Dihydroxyacetone was prepared by oxidising glycerol through the agency of *Acetobacter suboxydans*[1]. The dihydroxyacetone was isolated from the culture medium according to the directions of Bertrand [1898]. The dimeric compound was purified by washing with alcohol and then distilled at a pressure of 0·05 mm. [Fischer and Mildbrand, 1924]. The monomeric form thus obtained was washed with anhydrous alcohol-ether mixture (1 : 1) to remove traces of glycerol and dried over phosphorus pentoxide; M.P. 72–80° (uncorr.).

Table VI shows the results of the estimations.

Table VI.

1 cc. *N*/10 I≡4·5 mg. dihydroxyacetone.

Mg. dihydroxyacetone		
Used	Found	% yield
6·00	5·79	97
3·00	2·95	98
1·50	1·47	98
0·751	0·755	100
0·375	0·380	101

[1] We wish to express our thanks to Prof. A. J. Kluyver of Delft for kindly supplying us with a culture of *Acetobacter suboxydans*.

(4) dl-*Glyceraldehyde.*

This substance was prepared from glycerol by the synthesis of Wohl [1898]. The details described by Witzemann [1914] were followed, except for the preparation of β-chloropropionacetal, in which the conditions of Reeves [1927], which give a superior yield, were employed; M.P. 139–40° (uncorr.). The solutions were allowed to stand in the presence of toluene and a trace of sulphuric acid for at least 48 hours before estimation, in order that the compound should pass over to the monomeric form. Table VII shows the results of the bisulphite titrations.

Table VII.

1 cc. $N/10$ I$\equiv$4·5 mg. glyceraldehyde.

Mg. glyceraldehyde		
Used	Found	% yield
5·86	5·66	98
5·00	4·89	98
2·93	2·78	95
1·47	1·48	101
0·733	0·726	99
0·365	0·354	97

V. Determination of the constants K and F and examples of the estimation of mixtures of pyruvic acid with methylglyoxal or acetaldehyde.

The determinations of the constant K with quantities of the alkali-labile carbonyl compounds varying between 6·0 and 0·2 mg. in each case are summarised in Table VIII.

Table VIII.

		Mean
Acetaldehyde	0·015, 0·007, 0·006, 0·006, 0·006, 0·006	0·008
Glyceraldehyde	0·13, 0·060, 0·030, 0·020, 0·020	0·05
Dihydroxyacetone	0·17, 0·12, 0·17, 0·15, 0·13	0·15
Methylglyoxal	0·014, 0·019, 0·009, 0·031, 0·010, 0·009, 0·010, 0·011, 0·042	0·014
Acetoacetic acid	0·190, 0·068, 0·048, 0·025, 0·029	0·07

The experimental error in these determinations is large owing to the small titrations after the alkali treatment. This however does not greatly affect the accuracy of the estimation, since f is small compared with F.

The figures for acetoacetic acid are based on the initial concentration determined by the Van Slyke method (see section VII).

The estimations of the percentage of pyruvic acid remaining after heating with alkali are somewhat inconsistent. We have been unable to find any explanation of this inconsistency. Triplicate titrations of samples of the same solution after heating with alkali show considerable variations.

Table IX gives examples of the results obtained.

Table IX.

Mg. pyruvic acid (in 5 cc.)	F		
6·0	0·914	0·905	0·992
2·8	0·945	0·960	0·755
1·4	0·869	0·903	0·949
0·7	0·897	0·955	0·940
0·35	0·906	0·926	0·104

The mean of a very large number of determinations gives $F = 0{\cdot}93$.

The values of F obtained for hydroxymaleic acid are shown in Table X. The initial concentration of hydroxymaleic acid is based on the weight taken and not on the bisulphite titration (see section VII).

Table X.

Mg. hydroxymaleic acid (in 5 cc.)	F
7·93	0·82
3·97	0·87
1·98	0·81
0·99	0·91
0·50	0·93

Table XI shows the results obtained in the estimation of mixtures of pyruvic acid with methylglyoxal and acetaldehyde.

Table XI.

Mg. used			Titrations expressed in terms of cc. $N/10$ I	Mg. found		
Pyruvic acid	Methyl-glyoxal	Acetal-dehyde		Pyruvic acid	Methyl-glyoxal	Acetal-dehyde
1·64	—	0·63	$T_1 = 0{\cdot}661$ $T_2 = 0{\cdot}357$	1·67	—	0·62
1·64	—	1·27	$T_1 = 0{\cdot}911$ $T_2 = 0{\cdot}345$	1·61	—	1·19
1·64	0·96	—	$T_1 = 0{\cdot}712$ $T_2 = 0{\cdot}341$	1·59	1·00	—
1·64	1·91	—	$T_1 = 1{\cdot}039$ $T_2 = 0{\cdot}346$	1·64	1·92	—

VI. Examples of the recovery of aldehydes and ketones added to tissues.

Technique.

10·0 g. quantities of the fresh rat tissue were finely chopped. An aliquot of a standard solution of the aldehyde or ketone under test was added, and after about 30 seconds' stirring, 5 cc. of 50 % trichloroacetic acid were added. The mixture was ground for a few minutes with sand and squeezed through muslin. The residue was extracted three or four more times by grinding with small quantities of water and again squeezing through muslin. The filtrate was where necessary (*viz.* in the methylglyoxal estimation) adjusted to about p_H 2 by the addition of N NaOH, made up to 100 cc. and re-filtered. 5 cc. aliquots

were then estimated in the usual manner. Allowance is made in each case for a tissue blank determined at the same time.

Table XII summarises the results.

Table XII.

Tissue	Substance added	Quantity added (mg.)	Quantity found	% recovery
Muscle	Pyruvic acid	66·7	64·8	97·1
,,	,,	7·22	6·96	96·4
Liver	Methylglyoxal	44·1	38·8	88·0
,,	,,	8·75	5·56	63·6
Muscle	,,	18·4	13·5	73·4
,,	Acetaldehyde	12·4	4·34	35·0
,,	Glyceraldehyde	44·6	44·2	99·1
Liver	,,	44·6	43·7	98·0
,,	Dihydroxyacetone	70·0	69·0	98·6
,,	,,	7·61	7·28	95·6

Tissue blanks.

10·0 g. rat-liver ≡ 0·915, 0·474, 0·332 cc. $N/10$ *I*
,, muscle ≡ 0·336, 0·457, 0·161 ,,

Considering the experimental difficulty of carrying out a quantitative extraction of the tissues and at the same time keeping the volume of the extract as small as possible, it may be assumed that the recovery of pyruvic acid and of the trioses is practically quantitative.

There are several indications in the literature that dilute solutions of methylglyoxal react in the cold with substances likely to be present in animal tissues. Thus Neuberg and Kobel [1927] showed that this keto-aldehyde reacted with amino-acids forming "melanin" together with CO_2, NH_3 and acetaldehyde. Müller and Pechmann [1889] and also Pinner [1902; 1905] showed that phenylglyoxal readily condensed with ammonia. The reaction of phenylglyoxal with a constituent or constituents of the pancreas was also studied by Giršavičius [1930].

The failure to effect a quantitative recovery of acetaldehyde from muscle may be connected with the observation of Fabre [1925] that while a deproteinised blood-filtrate yields about 0·77 mg. of acetaldehyde per litre when this substance is distilled from the liquid in a current of nitrogen, the same filtrate only yields 0·48 mg. per litre when allowed to stand in the same closed air-space as the Nessler's reagent used for estimating it.

The tissue blanks observed with rat-muscle may be accounted for, at least in part, by the presence of pyruvic acid and methylglyoxal in the filtrates obtained under these conditions [Case and Cook, 1931]. We have made repeated attempts to isolate and identify the substance or substances responsible for the bisulphite-binding capacity of trichloroacetic acid extracts of ox-liver without success. We are at present only able to report that pyruvic acid is not amongst them.

VII. The behaviour of β-ketonic acids and acetone.

An attempt to apply the method to acetoacetic and oxaloacetic acids has shown that these substances cannot be estimated with any great accuracy. Our observations seem however to be worth recording, since they may be of use in a qualitative or semiquantitative manner. The reason for the inapplicability of the method to these substances is twofold. In the first place the bisulphite compounds appear to be sufficiently unstable in acid solution to render the first end-point ill-defined. Secondly a slow formation of iodoform occurs when the solutions of these substances stand in the presence of iodine and sodium bicarbonate. This renders the second end-point ill-defined. We record in Tables XIII and XIV the results of a series of determinations, taking as the end-points a blue coloration which lasted for about 15 seconds.

Table XIII.

Mg. acetoacetic acid		
Used	Found	% of theory*
7·11	6·16	86·6
3·56	3·09	86·7
1·78	1·54	86·5
0·889	0·778	87·4
0·444	0·397	89·4

* Calculated on the basis of 1 cc. $N/10$ I≡5·1 mg. acetoacetic acid.

Table XIV.

Mg. hydroxymaleic acid		
Used	Found	% of theory*
7·93	6·51	82·2
3·97	3·28	82·6
1·98	1·62	81·8
0·991	0·892	90·4
0·496	0·427	86·1

* Calculated on the basis of 1 cc. $N/10$ I≡6·6 mg. oxaloacetic acid.

The sodium acetoacetate solution was prepared free from acetone and ethyl acetoacetate by the method of Shaffer [1921] and standardised by the method of Van Slyke [1917]. The bisulphite titrations and Van Slyke determinations were both made within an hour of the removal of the solution from aeration flask (aeration time was 20 hours).

The oxaloacetic acid (hydroxymaleic acid) was prepared by the method of Simon [1903] from ethyl oxaloacetate and recrystallised from ethyl acetate by the addition of benzene. The substance softened at 145° and decomposed at 156°. The purity was checked by titration with alkali.

30·38 mg. required 4·58 cc. $N/10$ NaOH (phenolphthalein)
Calculated titre = 4·60 ,, ,,

Only freshly prepared solutions were used.

Acetone cannot be estimated owing to the instability of the bisulphite compound in solution.

VIII. Interfering substances.

Reduced glutathione, proteins, glycogen and urea do not interfere with the titration. After heating with alkali however, reduced glutathione solutions have a bisulphite-binding capacity which is probably due to the presence of pyruvic acid which is known to occur amongst the alkaline decomposition products of cystine [see *e.g.* Friedmann, 1903]. For example, 6·85 mg. of glutathione, after treating with alkali, required 0·0385 cc. *N*/10 I.

Unsaturated substances interfere to a small extent in relatively high concentrations. Thus

50 mg.	of maleic acid	≡ 0·070 cc.	*N*/10 I
50 ,,	fumaric acid	≡ 0·017	,,
50 ,,	cinnamic acid	≡ 0·026	,,

Bile has a slight bisulphite-binding capacity; *e.g.* 5 cc. of fresh ox-bile took 0·070 cc. *N*/10 I.

Commercial yeast nucleic acid gives a small titre. Thus 50 mg. took 0·022 cc. *N*/10 I.

The behaviour of glucose is remarkable. Neutral solutions of glucose (Kahlbaum) yield a slight titre. 5 cc. aliquots containing the below-mentioned amounts of glucose took the amounts of iodine indicated.

Mg. glucose	cc. *N*/10 I
460	0·15
50	0·05
10	0·01

The end-points are very ill-defined. After heating with alkali the titre is not sensibly increased. Fructose behaves similarly.

In the presence of ammonium salts or amino-acids, however, the titre of glucose is greatly increased. This is only the case if the solution is approximately neutral prior to the addition of the bisulphite. The presence of an amount of acid or base, small compared with the amount of acid represented by the bisulphite, reduces the titre to a value of the same order as it has in the absence of ammonium salts or amino-acids. For example 10 mg. of glucose in the presence of 5 mg. of ammonium chloride have a titre of the order of 0·7 cc. *N*/10 I. In the presence of *N*/100 HCl or *N*/100 NaOH this titre is reduced to 0·01 cc.

It is hoped to publish an investigation of this remarkable effect in a later communication.

Summary.

1. A method of estimating pyruvic acid and methylglyoxal depending on the capacity of these substances to form compounds with bisulphite is described. The minimum amount of these substances to which the method is sensitive is 0·2 mg. of either substance in 5 cc.

2. From the fact that methylglyoxal and other aldehydes and ketones are destroyed by heating in alkaline solution while pyruvic acid and certain other ketones are not, a method has been devised by means of which the components of a mixture of one of the former with one of the latter may be determined.

3. The method is extended to the determination of glyceraldehyde, dihydroxyacetone and acetaldehyde.

4. The effect of compounds containing an ethylene linkage or a sulphydryl group has been studied. In general these compounds do not affect the estimation.

5. The methods of preparation of the carbonyl compounds studied are indicated and the question of some polymerides of pyruvic acid is discussed.

6. The method for the recovery and estimation of some of the above compounds in the presence of animal tissues is described.

We wish to thank Sir Frederick Hopkins for his kind encouragement during the course of this research. One of us (R. P. C.) is indebted to the Chemical Society for a grant for the purchase of chemicals.

REFERENCES.

Bertrand (1898). *Compt. Rend. Acad. Sci.* **126**, 984.
Case and Cook (1931). *Biochem. J.* **25**, 1319.
Clausen (1922). *J. Biol. Chem.* **52**, 263.
Cook (1930). *Biochem. J.* **24**, 1526.
Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 127.
Fabre (1925). *Bull. Soc. Chim. Biol.* **7**, 429.
Fischer and Mildbrand (1924). *Ber. deutsch. chem. Ges.* **57**, 707.
—— and Taube (1924). *Ber. deutsch. chem. Ges.* **57**, 1502.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Friedmann (1903). *Beitr. chem. Physiol. Path.* **3**, 1.
Giršavičius (1930). *Biochem. J.* **24**, 446.
de Jong (1901). *Rec. trav. chim.* **20**, 365.
—— (1902). *Rec. trav. chim.* **21**, 299.
Kuhn and Heckscher (1926). *Z. physiol. Chem.* **160**, 116.
Müller and Pechmann (1889). *Ber. deutsch. chem. Ges.* **22**, 2556.
Neuberg and Kobel (1927). *Biochem. Z.* **185**, 477.
Pinner (1902). *Ber. deutsch. chem. Ges.* **35**, 4131.
—— (1905). *Ber. deutsch. chem. Ges.* **38**, 1531.
Quastel and Wooldridge (1929). *Biochem. J.* **23**, 115.
Reeves (1927). *J. Chem. Soc.* 2477.
Ripper (1900). *Monatsh. Chem.* **21**, 1079.
Shaffer (1921). *J. Biol. Chem.* **47**, 433.
Simon (1903). *Compt. Rend. Acad. Sci.* **137**, 855.
Van Slyke (1917). *J. Biol. Chem.* **32**, 455.
Wendel (1932). *J. Biol. Chem.* **94**, 717.
Wieland (1923). *Liebig's Ann.* **436**, 229.
Witzemann (1914). *J. Amer. Chem. Soc.* **36**, 1908.
Wohl (1898). *Ber. deutsch. chem. Ges.* **31**, 1796, 2394.
Wolff (1899). *Liebig's Ann.* **305**, 154.
—— (1901). *Liebig's Ann.* **317**, 1.

CCXII. THE 2:4-DINITROPHENYLHYDRAZONES OF SOME KETONIC ACIDS OF BIOLOGICAL IMPORTANCE.

By FRANCIS PAUL CLIFT[1] and ROBERT PERCIVAL COOK[2].

From the Biochemical Laboratory, Cambridge.

(*Received October 2nd, 1932.*)

During the course of some experiments on the carbohydrate metabolism of the liver it was found necessary to prepare the derivatives of certain compounds which were thought to be of possible biological importance. Among numerous derivatives prepared were the 2 : 4-dinitrophenylhydrazones of mesoxalic, oxaloacetic and acetoacetic acids. As these compounds do not appear to have been described hitherto in the literature we record below our observations on their preparation, which requires some care, and their properties. We have also devised a quantitative method by which their identity may be verified.

Mesoxalic acid 2:4-dinitrophenylhydrazone.

Mesoxalic acid was prepared from diethylmalonate by the method of Curtiss [1906]. The acid was converted into the sodium salt and the latter purified by repeatedly precipitating it from its aqueous solution with alcohol.

Slightly more than the molecular equivalent of 2 : 4-dinitrophenylhydrazine dissolved in boiling 2*N* HCl (0·5 g. in 30 cc.) was added to an aqueous solution of sodium mesoxalate. The hydrazone was immediately precipitated in hair-like pale yellow needles. Sufficient strong hydrochloric acid was then added to render the solution 2*N* and the mixture allowed to stand overnight. The hydrazone was filtered off, washed with 2*N* HCl, and dried *in vacuo* over sulphuric acid. The yield was practically quantitative. The substance is appreciably soluble in water (1 part dissolves in approximately 400 of water at room temperature), but is almost insoluble in 2*N* HCl. It may be conveniently recrystallised by dissolving it in water and adding strong hydrochloric acid. The melting-point is 202° (uncorr.) unaltered by further recrystallisation. (Found N 18·73 %; Calculated N 18·79 %[3].)

The precipitation of the hydrazone occurs even in very dilute solution. Thus a precipitate is formed when 17 mg. of 2 : 4-dinitrophenylhydrazine

[1] Reginald John Smith Research Student.

[2] Beit Memorial Research Fellow.

[3] This and subsequent analyses by Mr A. Colwel.

dissolved in 1 cc. of 2*N* HCl are added to 4 cc. of a solution containing only 1·5 mg. of mesoxalic acid.

The hydrazone dissolves in saturated sodium carbonate to give a light brown solution from which it can be reprecipitated by acidification with hydrochloric acid. It is readily soluble in ethyl acetate and is completely thrown out from its solution in this solvent by the addition of a small amount of light petroleum. It is slightly soluble in alcohol, almost insoluble in ether. Although it is a malonic acid derivative its solution can be safely heated to 100°. An aqueous solution of the substance, saturated at the boiling-point, was boiled under reflux for 1 hour. On acidifying with hydrochloric acid the hydrazone was recovered unchanged in melting-point. The melting-point of glyoxylic acid 2 : 4-dinitrophenylhydrazone, the formation of which might be expected under these conditions is given by Brady [1931] as 190°.

The substance differs from other 2 : 4-dinitrophenylhydrazones in giving no colour with alcoholic potassium hydroxide.

Acetoacetic acid 2 : 4-dinitrophenylhydrazone.

Acetoacetic acid was prepared as the sodium salt in aqueous solution and freed from ethyl acetoacetate and acetone by the method of Shaffer [1921]. The hydrazone was prepared as described above, except that the hydrazine was added as a supersaturated solution in 2*N* HCl at room temperature. The yield of crude product was 85 % of the theoretical figure based on an estimation of the strength of the acetoacetic acid solution by the method of Van Slyke [1917]. The substance was purified by dissolving it in saturated sodium carbonate solution (20 cc. per g. of material), filtering from a trace of insoluble material, and precipitating it by the addition of hydrochloric acid. The precipitate was dried *in vacuo* over sulphuric acid. Crystallisation was effected by dissolving it in the minimum quantity of ethyl acetate at room temperature, and adding ten times the volume of ligroin. The substance crystallises in parallel growths of brownish-yellow pointed needles. It melts somewhat indefinitely at 125° (uncorr.) with evolution of carbon dioxide and the formation of a red liquid. The melting-point was unchanged by further recrystallisation. (Found, N 19·62 %; Calculated, N 19·86 %.)

The hydrazone gives a dark brown solution in saturated sodium carbonate and can be precipitated therefrom by acidification. It is slightly soluble in alcohol but almost insoluble in ether. It is practically insoluble in water. The substance gives a red colour with alcoholic potassium hydroxide.

At no stage in its purification should it be heated, either alone or in solution. In solution in ethyl acetate or glacial acetic acid it undergoes decomposition when warmed, considerably below the boiling-points of these solvents, yielding carbon dioxide and acetone 2 : 4-dinitrophenylhydrazone. The latter was obtained in a state of purity simply by two crystallisations of acetoacetic acid 2 : 4-dinitrophenylhydrazone from boiling ethyl acetate. The derivative melted sharply at 126° (uncorr.). The melting-point of the acetone

hydrazone prepared from acetone and once recrystallised from ethyl acetate was 126°. A mixed melting-point of the two specimens showed no change from this figure. The crystals of both specimens were short rhombic prisms. (Found, N 23·71 and 23·81 %; Calculated, N 23·53 %.)

This hydrazone gives a red colour with alcoholic potassium hydroxide.

Oxaloacetic acid 2:4-dinitrophenylhydrazone.

The oxaloacetic acid used was the maleinoid form (melting-point 150° approx.) prepared by the method of Simon [1903]. The conditions described above for the preparation of the acetoacetic acid derivative were employed. The hydrazone separates slowly in glistening rhombic plates. The substance is thus obtained in a pure state, as judged by constancy of melting-point after recrystallisation, by the nitrogen content, and by the alkali titration figure (see below). (Found, N 17·91 %; Calculated, N 17·94 %.)

The hydrazone dissolves in saturated sodium carbonate to give a brown solution from which it can be reprecipitated by acidification. It is readily soluble in ethyl acetate and can be caused to crystallise from this solvent by the addition of ligroin or benzene. It is slightly soluble in alcohol but almost insoluble in ether. One part of the substance dissolves in about 140 parts of water at room temperature. It can be caused to crystallise from its saturated aqueous solution by adding enough strong hydrochloric acid to make the solution 2*N*.

It softens at 205° and melts at 211° with evolution of gas. Like the analogous acetoacetic acid derivative it decomposes when heated, although rather less readily. Thus drying the substance at 110° for 2 hours caused the melting-point to fall to 208°. If a saturated solution of the substance is made in boiling ethyl acetate, crystallisation does not occur on cooling. The addition of light petroleum however causes the separation of a yellow semicrystalline precipitate. Titration with alkali indicates that partial decomposition into pyruvic acid 2 : 4-dinitrophenylhydrazone has occurred. 4·26 mg. of the product required 2·47 cc. of *N*/100 NaOH. The calculated titres for oxaloacetic acid hydrazone and pyruvic acid hydrazone are 2·64 and 1·63 cc. respectively.

Characterisation of small quantities of the 2:4-dinitrophenylhydrazones of ketonic acids by acidimetry.

In spite of the intense colour of these substances it is possible to dissolve them in excess of standard alkali and titrate the solution with standard acid, provided that an indicator is chosen the colour change of which is not masked by the colour change due to the derivatives themselves. Notwithstanding its low p_K, we have found that bromothymol blue gives satisfactory end-points. This titration may be performed with small quantities of material (2 to 5 mg.) weighed on a micro-balance. In the identification of an unknown substance it affords a useful check on the nitrogen determination. Determinations of C and H are not of great value with these derivatives owing to the

fact that different compounds often have very nearly the same percentage composition with respect to these elements.

Examples of these titrations are shown in the table below. In each case the quantity of the substance indicated was weighed out, dissolved in 5 cc. of $N/100$ NaOH, and titrated with $N/100$ acid.

Microtitrations of 2 : 4 dinitrophenylhydrazones.

Parent acid	Weight taken (mg.)	cc. of $N/100$ NaOH	
		Found	Calculated
Pyruvic	3·74	1·42	1·39
Acetoacetic	4·02	1·44	1·42
Oxaloacetic	3·06	1·97	1·96
Mesoxalic	3·07	1·96	1·96

Summary.

1. The preparation and properties of the 2 : 4-dinitrophenylhydrazones of mesoxalic, acetoacetic and oxaloacetic acids are described.

2. A method is given by which these derivatives may be titrated as acids and which may be used in their identification.

We wish to thank Sir Frederick Hopkins for his kind encouragement. One of us (R. P. C.) is indebted to the Chemical Society for a grant for the purchase of chemicals.

REFERENCES.

Brady (1931). *J. Chem. Soc.* 752.
Curtiss (1906). *Amer. Chem. J.* **35**, 478.
Shaffer (1921). *J. Biol. Chem.* **47**, 433.
Simon (1903). *Compt. Rend. Acad. Sci.* **137**, 855.
Van Slyke (1917). *J. Biol. Chem.* **32**, 455.

CCXIII. TRIOSE DEHYDROGENASE. I.

By FRANCIS PAUL CLIFT[1] and ROBERT PERCIVAL COOK[2].

From the Biochemical Laboratory, Cambridge.

(*Received September 27th, 1932.*)

The existence of the trioses as intermediary compounds in mammalian carbohydrate metabolism has often been postulated. Since *dl*-glyceraldehyde was first synthesised by Wohl [1898] a number of investigations have been made on the changes which it may undergo in the animal body. References to these are given by Stöhr [1932]. It appears that the compound is capable of complete oxidation in the animal body, and that the liver has the power of converting it into lactic acid, glycogen or hexose. The production of *d*-sorbose from *dl*-glyceraldehyde [Embden, Schmitz and Wittenberg, 1913] is readily explicable on stereochemical grounds. This observation is of importance as it appears to indicate that glyceraldehyde may first be converted into dihydroxyacetone under the conditions prevailing in the liver cells.

An even more extensive series of investigations has been carried out on the behaviour of dihydroxyacetone in mammalian metabolism. The liver can convert dihydroxyacetone into glucose or glycogen. This triose, in contradistinction to glyceraldehyde, has the power of relieving insulin convulsions [Reeves and Hewitt, 1926]. It is not however antiketogenic.

Most of the work hitherto carried out on the trioses has demonstrated only their capacity to play a part in the synthesis of hexoses or glycogen, or to undergo internal oxidation-reduction to lactic acid. The possibility that the trioses might arise during carbohydrate catabolism and undergo direct oxidation has not been seriously entertained. The fact that dihydroxyacetone is autoxidisable in aqueous solution has long been known, and Wind [1925] has studied the influence of heavy metal ions on the autoxidation of both trioses in alkaline phosphate buffer. Euler, Eriksson and Brunius [1928] showed that dihydroxyacetone solutions were capable of reducing methylene blue in the presence of phosphate buffer. As far as we are aware the only observation hitherto made on the catalysis of triose dehydrogenations by an enzyme of mammalian origin is that of Lebedev [1926] who showed that the purified Schardinger enzyme preparation from milk catalysed this reaction.

We have studied the catalytic action of a liver extract prepared by the method of Harrison [1931] on the trioses, on acetaldehyde and on hypoxanthine in the presence of methylene blue.

[1] Reginald John Smith Research Student. [2] Beit Memorial Research Fellow.

Experimental.

The reduction velocity as a function of substrate concentration.

In order to establish the identity or non-identity of the enzymes responsible for the dehydrogenations studied by the "competition" method, it was necessary to determine the optimum concentration of each substrate. In the following experiments an enzyme preparation which had been dialysed at 0° nearly free from ammonium sulphate was used. In general 4 to 6 2-litre changes of distilled water were used in the course of 48 to 72 hours. At the end of this time the last dialysate gave only an opalescence with barium chloride solution. 10 g. of the enzyme powder yielded about 40 cc. of solution. Since the enzyme solution was deeply pigmented, the time for apparently complete reduction of the methylene blue was observed. The velocities recorded in the following curves and tables are the reciprocals of the reduction times in minutes multiplied by 100. In every case the contents of the Thunberg tubes consisted of 1 cc. of the enzyme solution and 0·5 cc. of a solution of 1 part of methylene blue in 5000 parts of phosphate buffer. The phosphate buffer was Clark and Lubs p_H 7·2 buffer made up to be 10 times as concentrated as that given by Clark *viz.* 0·5 *M* with respect to phosphate. When diluted with 1 volume of water and 2 volumes of enzyme solution the resulting mixture had a p_H of 7·1 determined electrometrically. The total volume of the contents of the Thunberg tube was made up to 2·0 cc. with the substrate solution and water where necessary. The reduction times were in every case measured at 37°. The reduction time for the enzyme solution alone under these conditions is of the order of 5 hours.

dl-*Glyceraldehyde.* Crystalline glyceraldehyde prepared as indicated in a previous paper [Clift and Cook, 1932] was used in these experiments. Owing to the sparing solubility of the dimeric form in water, the strong solutions

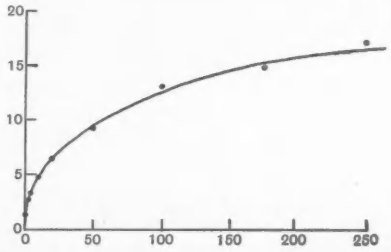


Fig. 1. Initial reaction velocity as a function of glyceraldehyde concentration. Ordinates: relative velocity. Abscissae: glyceraldehyde concentration in millimols. per litre.

necessary were made by dissolving the glyceraldehyde in the minimum amount of water and allowing it to stand at room temperature for several days in the presence of toluene (to prevent infection) until the water could be removed *in vacuo* over sulphuric acid without the glyceraldehyde crystallising out. Although

glyceraldehyde reduces methylene blue in the presence of phosphate buffer, and in the absence of enzyme, control experiments showed that the velocity under these conditions was negligible compared with that of the enzymic reduction. The Michaelis curve for *dl*-glyceraldehyde is shown in Fig. 1. Within the limits of experimental error this curve is represented by the well-known equation

$$v = \frac{Vx}{K_m + x},$$

where v = initial velocity of the reaction,
x = concentration of substrate,
V = velocity of reaction when x is large,
K = Michaelis constant of the system.

Dihydroxyacetone. Freshly prepared solutions of monomeric dihydroxyacetone (prepared as described by Clift and Cook [1932]) were used in every case. Under the conditions described above the velocity of methylene blue reduction by dihydroxyacetone in the absence of the enzyme is not negligible compared to that of the enzymic reaction. Fig. 2 shows the Michaelis curve for methylene blue reduction in the presence of the enzyme. The lower curve shows the relationship between the non-enzymic reduction velocity and the concentration of dihydroxyacetone. Even when allowance is made for the non-enzymic reduction velocity, the curve does not fit the Michaelis equation, the velocity in high substrate concentrations being higher than this equation indicates.

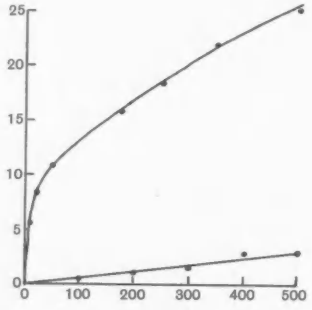


Fig. 2.

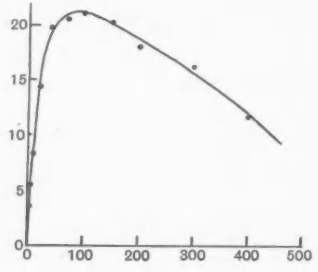


Fig. 3.

Fig. 2. Initial reaction velocity as a function of dihydroxyacetone concentration. Ordinates: relative velocity. Abscissae: dihydroxyacetone concentration in millimols. per litre. Upper curve: in the presence of the enzyme. Lower curve: in the absence of the enzyme.

Fig. 3. Initial reaction velocity as a function of acetaldehyde concentration. Ordinates: relative velocity. Abscissae: acetaldehyde concentration in millimols. per litre.

Acetaldehyde. The relationship of the reduction velocity to the concentration of acetaldehyde is illustrated in Fig. 3. This curve shows that the aldehyde dehydrogenase is inhibited by high substrate concentrations. Acetaldehyde, added in high concentrations to the enzyme preparation causes the appearance

of a precipitate. Since a certain amount of acetaldehyde boils off when the Thunberg tubes are evacuated, no quantitative conclusions can be drawn from the shape of the curve in high concentrations of acetaldehyde. It will be observed that the aldehyde dehydrogenase present in the liver extract has a very different velocity-substrate concentration curve from the Schardinger enzyme studied by Dixon. The optimum aldehyde concentration is 0·1 *M* and the concentration giving half the maximum reduction velocity is 0·013 *M*.

Hypoxanthine. The reduction velocities with various concentrations of hypoxanthine are shown in Table I.

Table I.

Concentration (millimols. per litre) of hypoxanthine	Reduction velocity
1·7	8·3
1·4	8·9
1·0	11·0
0·68	10·9
0·34	10·9
0·17	11·1

The identity of the dehydrogenases.

An attempt to ascertain whether the same or different enzymes are responsible for the dehydrogenations of glucose, the trioses, acetaldehyde and hypoxanthine has been made by allowing the preparation to act on these substrates taken in pairs each at the optimum or nearly optimum concentrations. According to the generally accepted theory of enzyme action, if the same enzyme is responsible for the oxidation of two substrates the reduction velocity for a mixture of the two substrates in optimum concentration should lie between the reduction velocities for the two substrates separately. If different enzymes are responsible, then the velocity for a mixture should be the sum of the velocities for the two components separately. In the results of such experiments shown in Table II, the above conditions were used. The concentrations of the various substrates were: glucose 0·25 *M* [Harrison, 1931], dihydroxyacetone 0·2 *M*, glyceraldehyde 0·15 *M*, acetaldehyde 0·1 *M*, hypoxanthine 0·0007 *M*. Since the velocity-substrate concentration curves for the trioses show that the reaction velocity continually increases with increasing concentration of the substrate, it is to be anticipated that if the same enzyme is responsible for both dehydrogenations the velocity for the dehydrogenation of the mixture of the ketose and the aldose will be somewhat greater than the mean of the velocities for each separately. Table II shows this to be the case. Hence it may be tentatively concluded that the same enzyme is responsible for catalysing both reactions.

The reduction velocity for a mixture of glucose with either triose is slightly less than the sum of the velocities for the components separately. This discrepancy is probably not due to experimental error since a number of experiments gave figures similar to those quoted. Since, however, the velocity for the mixture is considerably greater than the velocity for either glucose alone

Table II.

Substrate	Reduction time mins.	Observed velocity	Sum of observed velocities
Glucose	11·9	8·4	
Glyceraldehyde	8·1	12·4	
Glucose + glyceraldehyde	5·9	17·0	20·8
Glucose	14·3	7·0	
Dihydroxyacetone	7·6	13·2	
Glucose + dihydroxyacetone	5·5	18·2	20·2
Glucose	9·8	10·2	
Acetaldehyde	3·2	31·2	
Glucose + acetaldehyde	3·2	31·2	41·4
Glucose	11·1	9·0	
Hypoxanthine	5·6	17·9	
Glucose + hypoxanthine	3·9	25·6	26·9
Glyceraldehyde	7·4	13·5	
Dihydroxyacetone	6·3	15·9	
Glyceraldehyde + dihydroxyacetone	4·9	20·4	29·4
Glyceraldehyde	7·4	13·5	
Acetaldehyde	3·0	33·3	
Glyceraldehyde + acetaldehyde	3·1	32·2	46·8
Glyceraldehyde	7·4	13·5	
Hypoxanthine	3·8	26·4	
Glyceraldehyde + hypoxanthine	2·6	38·5	39·9
Dihydroxyacetone	7·7	13·0	
Acetaldehyde	4·0	25·0	
Dihydroxyacetone + acetaldehyde	4·5	22·2	38·0
Dihydroxyacetone	6·2	16·1	
Hypoxanthine	3·8	26·3	
Dihydroxyacetone + hypoxanthine	2·3	43·5	42·4
Acetaldehyde	3·6	27·8	
Hypoxanthine	5·6	17·9	
Acetaldehyde + hypoxanthine	4·4	22·7	45·7

or a triose alone, it seems probable that different enzymes are responsible for the two catalyses. This conclusion is supported by the fact that the activity of the extract on glucose decreases by about 50 % in the course of 8 hours at room temperature, while no difference can be detected in the activity of the extract on the trioses under these conditions.

Methylene blue reductions carried out with mixtures of glucose or a triose with acetaldehyde show that the velocity with the mixture is the same as the velocity with acetaldehyde alone. This seems to indicate that acetaldehyde inhibits the glucose and the triose dehydrogenase. If this interpretation is not put upon the phenomenon, the alternative assumption that the enzymes responsible are identical, leads to contradictory results. Thus with the latter assumption one would argue that the aldehyde dehydrogenase and the dihydroxyacetone dehydrogenase are identical. Since hypoxanthine and acetaldehyde appear to compete for the same enzyme, the hypoxanthine dehydrogenase would be identical with the dihydroxyacetone dehydrogenase. But the competition experiment shows that this is not the case.

Corroborative evidence as to the non-identity of the aldehyde and triose dehydrogenases was obtained by a study of the oxygen uptakes in the Barcroft

differential manometer. Hypoxanthine and acetaldehyde show an oxygen uptake in the presence of the enzyme. The action of the enzyme preparation on the trioses, however, is merely to effect a partial inhibition of the autoxidation which these substances show in phosphate buffer. Harrison showed that the glucose dehydrogenase is anaerobic. It seems possible but unlikely that the same enzyme should be capable of activating certain hydrogen donators with respect to oxygen as the acceptor, while it is unable to do so with others. Thus the competition experiments indicate that the extract contains three distinct dehydrogenases—one acting on glucose, one on the trioses, the third on hypoxanthine and acetaldehyde.

Discussion.

The work of Neuberg and his school has shown that methylglyoxal is a probable intermediary between polysaccharides and lactic acid in mammalian metabolism. A probable main course of the oxidative degradation of carbohydrate in muscle is given by Toenniessen and Brinkmann [1930]. Very little is known at present of the mechanism of carbohydrate oxidation in other tissues. We regard the observation that an enzyme capable of activating the trioses as hydrogen donators exists in the liver as of importance in so far as it indicates that these substances may be involved in the oxidative breakdown of carbohydrate in that organ. This possibility is of course open to the objection that the appearance of these very reactive substances in liver metabolism has never been observed.

Our observation raises the interesting question as to what are the oxidation products of the two trioses when dehydrogenated by the enzyme. The similarity in the behaviour of the enzyme with respect to the two substrates indicates that the product may be the same in both cases. The enzyme possibly acts on one triose only or on a common enolic form. The fact that the relationship between the initial reaction velocity and the dihydroxyacetone concentration approximates to linearity in high substrate concentrations indicates that under these conditions the factor limiting the velocity is a non-enzymic reaction, thus lending support to this hypothesis. Lebedev [1926] showed that the oxidation product of glyceraldehyde by the Schardinger enzyme preparation was probably glyceric acid. Our observations agree with his in so far as the enzyme acts more rapidly on the aldose than on the ketose. Lebedev showed that his enzyme was incapable of bringing about a Cannizzaro transformation of glyceraldehyde into glycerol and glyceric acid. Work is now in progress with a view to deciding whether the liver enzyme is capable of bringing about this change and to identifying the products of the oxidation of the trioses by methylene blue. Preliminary experiments have shown that the oxidation is in both cases attended with the production of more acid than can be accounted for by the HCl liberated by the methylene blue which is reduced.

SUMMARY.

1. An extract made from ox-liver contains an enzyme capable of activating glyceraldehyde and dihydroxyacetone as hydrogen donators to methylene blue.

2. The question of the specificity of the enzymes present in the liver extract is discussed. It is concluded that in the liver extract studied there are three distinct dehydrogenases, one acting on glucose, another on the two trioses and the third on hypoxanthine and acetaldehyde.

We wish to thank Sir Frederick Hopkins for his kind encouragement. One of us (R. P. C.) is indebted to the Chemical Society for a grant for the purchase of chemicals.

REFERENCES.

Clift and Cook (1932). *Biochem. J.* **26**, 1788.
Embden, Schmitz and Wittenberg (1913). *Z. physiol. Chem.* **88**, 210.
Euler, Eriksson and Brunius (1928). *Svensk. Kem. Tidskr.* **40**, 163.
Harrison (1931). *Biochem. J.* **25**, 1016.
Lebedev (1926). *Z. physiol. Chem.* **160**, 97.
Reeves and Hewitt (1926). *J. Physiol.* **61**; *Proc.* xxxv.
Stöhr (1932). *Z. physiol. Chem.* **206**, 211.
Toenniessen and Brinkmann (1930). *Z. physiol. Chem.* **187**, 137.
Wind (1925). *Biochem. Z.* **159**, 58.
Wohl (1898). *Ber. deutsch. chem. Ges.* **31**, 1796, 2394.

CCXIV. THE OSMOTIC PRESSURE OF GLOBIN.

By JEAN ROCHE, ANDRÉE ROCHE, GILBERT SMITHSON ADAIR and MURIEL ELAINE ADAIR[1].

From the Low Temperature Research Station and the Biochemical Laboratory, Cambridge.

(*Received September 10th, 1932.*)

The present communication records a series of measurements of the osmotic pressure of globin, the protein constituent of the haemoglobin molecule. A number of derivatives of haemoglobin have been investigated by osmometric methods, and it appears that in the cases of reduced and oxygenated haemoglobin, methaemoglobin [Adair, 1924; 1928] and carbon monoxide haemoglobin, which has been studied also by centrifugal methods [Svedberg and Fåhreus, 1926; Svedberg and Nichols, 1927] the molecular weight is a constant, equal to about 67,000.

The preparation of denatured globin from haemoglobin was described by Schulz [1898]. More recently, several methods of obtaining native globin have been suggested [Hill and Holden, 1926; Hamsik, 1930; Anson and Mirsky, 1930 and others]. Most of these involve the treatment of haemoglobin with acids and some organic solvent, whereby the pigment haematin is removed. Roche [1930] has found that at least three different globins are formed when haemoglobin is treated with acids, namely, native globin, fully denatured globin and an intermediate substance, insoluble at the isoelectric point, which has been termed paraglobin. Some measurements of the osmotic pressures of paraglobin solutions are also recorded in this paper.

I. Methods.

In this work, globin has been prepared by the methods of Hamsik [1930] and of Anson and Mirsky [1930]. A preparation of native globin (No. 6) was made by a slight modification of Hamsik's method, as follows. The haemoglobin of the ox, prepared by the ether and salt method of Adair [1925], was precipitated by the addition of acetone, crystals not being obtained. The precipitate was then treated with acetone containing oxalic acid, washed on a Büchner funnel with pure acetone and dissolved in water. The solution obtained was neutralised with 1 % ammonia, whereupon the paraglobin was precipitated, and the filtrate, containing native globin, was dialysed against distilled water. The preparation was carried out at 0° and yielded a slightly pigmented solution of globin.

[1] Beit Memorial Research Fellow.

Other preparations were made by the methods described by Anson and Mirsky. 0·1 *N* hydrochloric acid was added to an equal volume of 10 % ox-haemoglobin, both solutions being ice-cold, and the globin was precipitated by acetone containing 1 % of *N* hydrochloric acid. After washing the precipitate with pure acetone and then removing as much acetone as possible by suction and pressing between filter-papers, the globin was dissolved in water without previous drying in air, and, in the case of preparations 7 and 9, the solutions were slowly neutralised with 0·1 *N* sodium hydroxide, whereby most of the paraglobin was precipitated; this was finally removed by subsequent addition of 0·4 % ammonium sulphate to the filtrate. The preparation of globin from crystallised and uncrystallised horse-haemoglobin is described in Section V.

In the case of preparations of ox-globin Nos. 1, 2, 3 and 5, and of sheep-globin, the solution of protein, obtained after dissolving the acetone precipitate in water, was dialysed against an acetate buffer of p_H 5·6. As will be described later, the majority of such protein solutions appeared to consist of paraglobin at the end of the period of dialysis. Additional preparations of paraglobin were made by redissolving in a minimum quantity of acid the precipitate formed on neutralisation of solutions containing mixtures of globin and paraglobin.

In the case of ox-globin, we were able to obtain by the method of Anson and Mirsky protein solutions in which the presence of haematin was undetectable. Attempts to prepare sheep-globin by this method yielded in general appreciably pigmented solutions. In our hands, the method of Anson and Mirsky gave large yields of native globin, but we found the native protein to be relatively unstable, even when kept rigorously at 0°.

Osmotic pressure measurements were obtained by the method of Adair [1925], the protein solutions in rigid collodion membranes being equilibrated with standard buffer solutions at 0°. Series of experiments were carried out at two hydrogen ion concentrations, namely, p_H 7·7 and p_H 5·6, the two buffers being of the same ionic strength, 0·1. With this concentration of electrolytes, the pressures are not appreciably influenced by traces of carbon dioxide. After equilibrium had been attained, estimations were made of the concentration of the protein and, in certain cases, of the density of the protein and of the membrane potential, by a slight modification of the method of Loeb [1922].

The concentration of the protein was calculated from refractometric measurements [Adair and Robinson, 1930, 1] supported by Kjeldahl nitrogen determinations. The percentage of nitrogen in globin was calculated by deducting four g. molecules or 2525 g. of haematin, containing 224 g. nitrogen from 1 g. mol. or 67,000 g. of haemoglobin. The percentage of nitrogen in the dried haemoglobin of the ox is stated to be 17·7, but a reference to the original paper [Hüfner, 1887] showed that this figure is apparently based on a single analysis. It was deemed advisable to redetermine the nitrogen content by the method of Kjeldahl, and from the result obtained, 16·75 $\pm$ 0·15, it

was estimated that ox-globin contains 17·06 g. of nitrogen per 100 g. dry protein, and therefore the globin concentration is equal to the nitrogen multiplied by 5·862. The same factor has been used for sheep's globin. Schulz [1898] gives the value 16·89 % of nitrogen for horse-globin.

II. The stability of native globin.

According to Hill and Holden [1926] and Haurowitz and Waelsch [1929], globin is an unstable protein. We have made a number of tests to determine whether the degree of stability of the protein under the conditions of our experiments is sufficient for the purpose of osmometric measurements, in which the protein is equilibrated with buffer solutions at p_H 7·7 or p_H 5·6 for periods varying from 7 to 27 days at 0°.

(i) No diffusible protein breakdown products could be detected when the dialysates were tested by the xanthoproteic and the biuret reactions at the end of equilibration.

(ii) Haematin was added to the globin solutions after equilibration at p_H 7·7 and 5·6, and it was found by spectroscopic observations that in all cases the protein had retained the property, characteristic of native globin, of forming methaemoglobin, and subsequently reduced and oxy-haemoglobin on reduction and oxygenation. Since very small traces of native globin are required for this reaction, the spectroscopic tests are of only qualitative significance, as a proof that a certain amount of globin remains unchanged after equilibration.

(iii) Solutions of globin after equilibration at p_H 5·6 at 0° were brought to the isoelectric point by addition of alkali or phosphate buffer. Since native globin is soluble and para- and denatured globins are insoluble at the isoelectric point, the formation of a precipitate is a proof of alteration in the globin. The results for different solutions are recorded in Sections IV and V. It may be noted that in cases where experiments were continued for 18 to 28 days after the date of preparation, considerable amounts of precipitate were found, but in a number of experiments on dilute solutions, which were completed within 10 or 12 days, only traces of protein were precipitated. Attempts were made to estimate the amount of paraglobin formed by determining the total nitrogen and the soluble nitrogen after neutralisation, but the results of these analyses have not been recorded in the Tables because it is not yet known whether the precipitate formed is paraglobin only, or a mixture containing occluded native protein. In a few experiments, marked "much paraglobin" in Table III, about 70 % of the protein was apparently precipitated at the isoelectric point, but in other experiments the proportion precipitated varied from 0 to about 30 % of the total protein.

(iv) Further evidence concerning the stability of globin was obtained by osmometric experiments, in which the pressures were observed daily for periods from 7 to 25 days, and the osmometers were reset, in order to see if the

pressures returned to their original values. These experiments indicated that the stability of globin depends upon the species, the method of preparation, and the concentration of protein and of electrolytes. The best results were obtained with native globin from ox-blood at p_H 7·7. In Exp. 517, the pressure remained constant within 0·1 mm. for 7 days. At p_H 5·6 there were greater variations, probably owing to the transformation of native globin into paraglobin; the manometer readings diminished, instead of remaining constant, in experiments made with solutions containing more than 6 % of protein. The native globin of the horse is less stable; almost all the protein precipitated at p_H 7·7, and no estimate of osmotic pressure could be made, but at p_H 5·6 the pressures were constant within about 0·2 mm. Hg for a few days. A similar degree of constancy was observed in experiments on the paraglobin of the ox and the horse, unless the protein gelatinised, when the pressure fell more rapidly.

The reproducibility of the final pressures, obtained in a series of experiments with membranes of varying permeability and with different times for equilibration, is an additional criterion not only for the existence of osmotic equilibrium but also for the stability of the protein. The degree of reproducibility attainable in the case of dilute solutions of a fairly stable protein, such as serum-albumin, is illustrated in a series of 27 experiments in which the deviations from the mean pressure range from 0 to 4 %, recorded by Adair and Robinson [1930, 2]. In the series of 19 experiments with ox-globin at p_H 5·6, given in Table III, in 5 experiments the deviations from the mean pressure are less than 1 %; in 8 experiments, the deviations range from 1 to 4 %; in 4 experiments there are deviations of 4 to 10 %, and in 2 experiments from 10·5 to 13·1 %. It seems therefore that the preparation of ox-globin must have a certain degree of stability at p_H 5·6 as the deviations are not excessive, although they are considerably greater than those observed for serum-albumin. The measurement of the osmotic pressure of globin presents greater difficulties and the range of error is increased because the time available for obtaining a rigorous proof of equilibrium is limited by the fact that the protein may change into paraglobin.

III. The osmotic pressure of globin solutions at p_H 7·7.

Investigations of the osmotic pressure of globin at hydrogen ion concentrations varying from p_H 6·74 to p_H 8·26 are of special interest, as it has been shown by Roche [1930] that paraglobin and denatured globin are insoluble within this range. The pressures obtained should therefore be unaffected by such degradation products of globin.

Considerable advantage is obtained by the use of a buffer solution at approximately the isoelectric point of the protein, as at such a concentration of hydrogen ions the partial pressure due to the unequal distribution of diffusible ions, designated p_i, is very small, and the observed pressure is

approximately equal to p_g, the partial pressure of the protein ions. The isoelectric point of native globin has been determined by Roche [1929; 1930] by the method of cataphoresis, and found to be p_H 7·5 at 18°. Adair, Cordero and Shen [1929] have found that the p_H of mixtures of haemoglobin and bicarbonates increases from 0·014 to 0·018 if the temperature be diminished by 1°; it was inferred therefore that the isoelectric point of native globin should be about p_H 7·7 to p_H 7·8 at 0°, the temperature at which our osmotic experiments were carried out. This inference was supported by measurements of the membrane potential, recorded below.

The initial concentration of the buffer used was KH_2PO_4 0·00357 *M* and Na_2HPO_4 0·03214 *M*. The buffer value of phosphates is not very great at this hydrogen ion concentration, but previous experiments on haemoglobin have shown that if the ionic strength of the buffer be 0·1 or more, the observed osmotic pressures of dilute solutions of the protein are practically constant over a considerable range of p_H values.

In most of the experiments referred to below, in which no exceptional precautions were taken to exclude CO_2, the final percentage of phosphate ions in the bivalent form, calculated from hydrogen electrode measurements, was 85 % instead of 90 % as calculated from the original composition. Since one bivalent ion is equivalent to four univalent ions, the final value of the ionic strength of the buffers must have been from 0·1 to approximately 0·097.

Table I. *Native globin of the ox, osmotic pressures at* p_H *7·71–7·74 and 0°.*

Dialysate Na_2HPO_4, 0·03214 *M* + KH_2PO_4, 0·00357 *M*; ionic strength 0·10.

Preparation No.	Exp. No.	C	C_g	p	π_g	E
9 F	528	0·92	0·92	1·77	1·92	—
9 G	520	0·96	0·97	1·68	1·74	—
9 F	527	1·58	1·61	2·38	1·48	−0·04
7	496	1·58	1·61	2·42	1·50	—
9 F	526	1·72	1·75	3·00	1·72	—
9 G	519	1·91	1·94	2·91	1·50	—
9 J	529	2·12	2·16	3·23	1·50	−0·02
9 J	530	2·32	2·37	3·72	1·57	+0·03
9 G	518	2·77	2·85	3·89	1·37	−0·10
9 K	536	3·13	3·23	4·58	1·42	−0·15
7	495	3·42	3·53	4·46	1·27	−0·12
7	494	3·45	3·57	4·48	1·26	+0·3
9 G	516	3·94	4·09	5·22	1·28	—
9 G	517	4·04	4·20	5·26	1·26	−0·25
9 K	539	5·52	5·83	6·49	1·11	−0·42

C = g. dry protein per 100 cc. solution; C_g = g. dry protein per 100 cc. solvent; p = osmotic pressure in mm. Hg at 0°; $\pi_g = p/C_g$; E = membrane potential in millivolts.

The results obtained from a series of experiments on native globin solutions of concentrations ranging from 0·915 % to 5·52 % are given in Table I. Comparative experiments, carried out on carbon monoxide haemoglobin, made from the ox-haemoglobin solution that had been used for the preparations of globin numbered 9 F to 9 K are recorded in Table II.

Table II. *Carbon monoxide haemoglobin of the ox, osmotic pressures and membrane potentials at* p_H *7·75 and 0°.*

Dialysate Na_2HPO_4, 0·03214 *M* + KH_2PO_4, 0·00357 *M*, ionic strength 0·10.

Exp. No.	C	C_v	p	π_v	π_p	E observed	E calculated
549	0·89	0·89	2·35	2·63	2·62	—	−0·07
548	1·80	1·83	4·89	2·67	2·63	—	−0·14
547	3·01	3·10	8·82	2·85	2·79	−0·18	−0·24
546	4·95	5·19	15·02	2·90	2·81	−0·42	−0·40
545	8·57	9·34	29·62	3·18	3·01	−0·76	−0·72
544	14·26	16·55	58·30	3·53	3·23	−1·33	−1·27
543	16·51	19·62	75·40	3·84	3·48	−1·43	−1·50

Two methods for the tabulation of the protein concentrations have been adopted in Table I. Under the heading C, the results are given in g. per 100 cc. of solution; the chief advantages of this method are that C is directly proportional to the refractive index of the protein solution and the nitrogen content determined by volumetric analysis, and that it involves no speculations concerning the hydration of the protein. The corrected concentration C_v is calculated from measurements of C by formula (1).

$$C_v = C \times 100/(100 - V_1C) \qquad \text{.....(1).}$$

V_1 equals the volume occupied by 1 g. of dry protein *plus* the water of hydration combined with it. In the case of haemoglobin, in which about 0·2 g. of water appears to combine with 1 g. of protein [Adair and Robinson, 1931], the value of V_1 is about 0·965, and for the purpose of provisional calculations, the same value has been used for globin. The corrected concentration, C_v, is of service in the theoretical interpretation of the osmotic pressures.

A number of measurements of the membrane potentials of globin and haemoglobin are recorded in Tables I and II. The potentials obtained with haemoglobin are directly proportional to the protein concentration, as shown by the agreement between the observed values of E and these calculated by the empirical formula $E = k_eC_v$, where k_e is a constant equal to − 0·077. The negative sign shows that under these conditions the protein is acting as an anion.

In the experiments on globin, the conditions were not favourable for accurate measurements of membrane potentials. The observed potentials were small, and the available evidence is consistent with the assumption that at p_H 7·7 the protein is not far from the isoelectric point.

IV. The osmotic pressures of native globin and paraglobin of the ox at p_H 5·6.

One of the objects of this work was to compare the osmotic pressures of native globin and of paraglobin under the same conditions. Such a comparison is not possible at the isoelectric point, because paraglobin is insoluble. On the other hand, it is not possible to use solvents that are very far from neutrality, because native globin is then converted into paraglobin.

The determinations made previously by Roche [1930] indicated that at p_H 5·6 the solubility of paraglobin should be sufficient for the purpose of osmotic pressure determinations, and preliminary investigations made in this work showed that although native globin tends to change into paraglobin at p_H 5·6, the rate of change over a period of 7 to 14 days is not excessive, for in many of the experiments only a small fraction of the total protein was precipitated, when, at the end of equilibration, alkali was added to raise the p_H from 5·6 to the isoelectric point.

A list of the experiments made on preparations of native protein has been given in Table III. In the case of the preparations numbered 6 and 7, it appears that the pressure concentration ratio $p/C_v = \pi_v$ is a constant equal to 4·58, over the range of pressures from 4 to 50 mm. Of the 14 determina-

Table III. *Native globin of the ox, osmotic pressures at* p_H *5·64 and 0°.*

Dialysate sodium acetate, 0·10 *M* + acetic acid, 0·0105 *M*.
Preparation 6: method of Hamsik; preparations 7 to 9 *θ*, method of Anson and Mirsky.

Preparation No.	Exp. No.	C	C_v	p	π_v	E	Test for paraglobin
6	483	3·09	3·19	14·62	4·59	—	None
6	481	1·53	1·56	7·02	4·51	—	Trace
6	480	1·49	1·51	6·88	4·57	—	None
6	485	1·06	1·07	4·46	4·18	—	,,
6	484	0·95	0·96	4·51	4·72	—	,,
6	482	0·86	0·87	3·96	4·60	—	,,
7	486	10·18	11·30	48·80	4·32	+3·35	Some
7	487	10·18	11·30	53·30	4·72	+3·35	,,
7	488	7·54	8·13	37·40	4·60	+2·55	,,
7	489	5·79	6·13	28·60	4·66	+1·85	,,
7	490	3·33	3·42	15·46	4·52	+1·05	Little
7	491	1·64	1·67	7·59	4·55	—	Trace
7	492	0·84	0·85	4·10	4·83	—	Little
7	493	0·80	0·81	4·25	5·26	—	Some
9 G	521	1·51	1·53	7·22	4·73	—	Much
9 G	522	1·01	1·02	4·86	4·75	+0·05	,,
9 B	525	2·50	2·56	10·76	4·21	+0·57	Some
9 JA	531	2·08	2·13	9·63	4·52	+0·40	Much
9 *θ*	538	1·46	1·48	6·06	4·10	+0·45	,,

tions, 10 come within about 3 % of 4·58, and 4 show larger deviations, ranging from 6 to 15 %, but it is doubtful whether any significance can be attached to these deviations, because it was deemed advisable to finish the series rapidly, owing to the risk of denaturation of globin at p_H 5·6, and it is possible that the time allotted for equilibration and for determinations of capillarity corrections was insufficient to exclude the risk of experimental errors.

In the cases of preparations 9 G and 9 JA, in which the protein was equilibrated with buffer at p_H 7·7 before it was brought to p_H 5·6, the values for π_v agree with 4·58 within the limits of experimental error, but in the cases of preparations 9 B and 9 *θ*, there are deviations of 8 and 10 % respectively, which are probably due to the presence of paraglobin. It is interesting to note that preparation 6, made by the method of Hamsik, gives the same result as the other preparations, made by the method of Anson and Mirsky.

Before discussing the theoretical significance of the formula $p = 4{\cdot}58\, C_v$ for the osmotic pressure of globin, it is necessary to estimate the pressure, symbolised p_i, due to the excess of diffusible ions inside the membrane at p_H 5·6.

In the case of ideal or infinitely dilute solutions, the pressure p_i can be calculated by Donnan's equations. A different method has been developed [Adair, 1929] and a formula (No. 2) has been obtained which can be applied to non-ideal systems in which the activities of the diffusible salts are kept constant at different protein concentrations by equilibration with buffer solutions of the same composition.

$$p_i = RT \int_0^u m_p n_p du \qquad \text{.....(2).}$$

p_i = the pressure due to the excess of diffusible ions inside the membrane, expressed in mm. of mercury.

$$RT = 22{\cdot}4 \times 760 = 17{,}033 \text{ at } 0°.$$
$$u = E \times (F/1000RT) = E/23{\cdot}5 \text{ at } 0°,$$

where E = the membrane potential in millivolts.

$m_p n_p$ = the equivalent concentration of the protein acetate (the product of the molar concentration m_p and the valence n_p) in g. equivalents per litre of solvent.

The estimation of the equivalent concentration $m_p n_p$ has been discussed by Adair and Robinson [1930, 2], who have suggested that if E be small, under certain conditions the effects due to deviations from the ideal solution

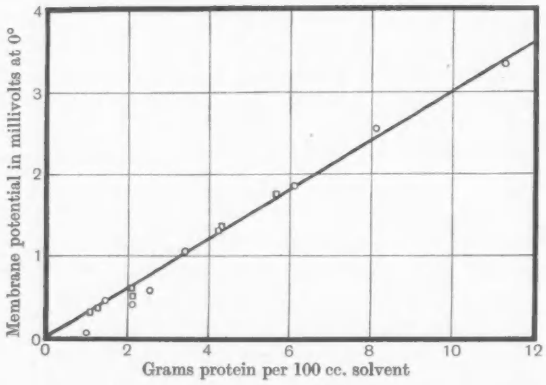


Fig. 1. Membrane potentials of globin solutions equilibrated with acetate buffer at p_H 5·64. Circles, native globin of ox. Squares, unfractionated globin and paraglobin.

laws may cancel out, and $m_p n_p$ may be equal to $-uJ$, where J is the sum of the concentrations of the ions in the dialysate, multiplied by the squares of their valencies. The ionic strength is equal to $\frac{1}{2}J$. A simple formula for p_i (No. 3) can be derived from formula (2), on the assumption that $m_p n_p = -uJ$.

This assumption is consistent with the experiments recorded in Table III and Fig. 1, which show that membrane potentials are directly proportional to the protein concentrations over the range investigated.

$$p_i = \tfrac{1}{2}RTJu^2 = 15{\cdot}4\,JE^2 = 15{\cdot}4\,Jk^2c_v^2 \qquad \text{.....(3).}$$

The term k in the formula numbered (3) is the ratio E/C_v, which must be a constant over the range of application of formula (3). In the observations recorded in Table III, $k = 0{\cdot}3$ and $J = 0{\cdot}2$.

It is probable that calculations of p_i based upon determinations of C_v and of the mean value of the ratio $E/C_v = k$ are more accurate than calculations based upon the individual values of the membrane potentials, which are subject to greater experimental errors than the measurements of protein concentration.

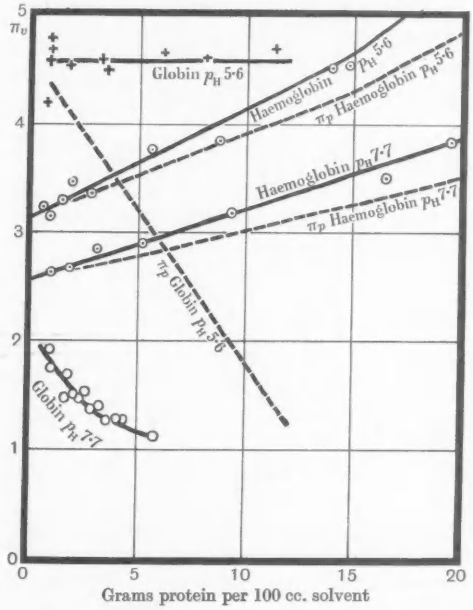


Fig. 2. Native globin and haemoglobin of ox. Pressure concentration ratios. Ordinate π_v and π_p in mm. of mercury at 0°. Abscissa C_v. Full lines, π_v; broken lines, π_p = ratio of partial pressure of protein ions and protein concentration. π_p is equal to π_v in solutions of globin at p_H 7·7.

The results recorded in Fig. 2 show that although the ratio π_v is practically constant, the ratio $\pi_p = p_p/C_v = (p - p_i)/C_v$ tends to diminish as the protein concentration is increased. This diminution is discussed in Section VII.

The results of measurements of the osmotic pressure of paraglobin are recorded in Table IV. The pressures observed are smaller than those given by native globin.

Table IV. *Unfractionated globin and paraglobin of the ox, osmotic pressures at* p_H *5·64 and 0°.*

Dialysate sodium acetate, 0·10 *M* + acetic acid 0·0105 *M*.

Preparations 1 and 2: unfractionated globin not dried before dialysis; preparation 4: unfractionated globin stored in dry state before dialysis; preparations 9 C and 9 L paraglobin.

Preparation No.	Exp. No.	C	C_v	p	π_v	E
1	364	1·25	1·27	4·51	3·57	+0·35
2	369	1·07	1·08	3·28	3·03	+0·30
4	391	2·05	2·09	6·81	3·26	+0·60
4	392	2·08	2·12	7·05	3·32	+0·5
4	389	4·06	4·23	11·60	2·75	+1·3
4	390	4·10	4·27	10·45	2·45	+1·35
9 C	523*	5·22	—	6·79	—	+1·35
9 C	524	2·58	2·65	4·56	1·72	—
9 L	537	5·38	5·68	9·61	1·69	+1·75

* Exp. 523, some protein in gelatinous form.

The osmotic pressures of preparations of unfractionated globin are given in Table IV, and it will be observed that the results are intermediate between those recorded for native globin and for the paraglobin separated by precipitation.

The osmotic pressures of fractionated native globin and paraglobin of the sheep have not yet been determined, but a preliminary series of measurements on unfractionated globin has been made, the results of which are recorded in Table V (prep. 3), in which the values of π_v diminished from 1·38 to

Table V. *Unfractionated globin of the sheep, osmotic pressures at* p_H *5·64 and 0°.*

Preparation No.	Exp. No.	C	C_v	p	π_v	α'
3	382	4·61	4·82	5·71	1·19	0·00185
3	383	2·28	2·33	2·89	1·24	0·00188
3	384	2·30	2·35	2·86	1·22	0·00187
3	385	3·35	3·47	4·28	1·23	0·00189
3	386	3·33	3·45	4·16	1·21	0·00189
3	387	1·15	1·16	1·56	1·34	0·00179
3	388	1·11	1·12	1·57	1·40	0·00187
5	433	3·31	3·42	5·70	1·67	0·00188

α' = the specific refraction increment (ref. Section VI).

1·17 as the protein concentration was increased. It appears that the unfractionated globin of the sheep gives lower osmotic pressures than any of the other forms of globin studied in this work.

For comparison with the experiments on globin, determinations of the osmotic pressure of haemoglobin at p_H 5·65 have been recorded in Table VI. The carbon monoxide and methaemoglobin solutions were prepared from the stock solution of haemoglobin used for the globin preparations numbered 9 G to 9 θ. The osmotic pressures are discussed in Section VII. The observed membrane potentials agree with those calculated by the empirical formula $E = kC_v$, given in the last column of Table VI, assuming that k is + 0·089 for CO-haemoglobin and + 0·119 for methaemoglobin.

Table VI. *Haemoglobin of the ox, osmotic pressure and membrane potentials at* p_H *5·65 and 0°.*

Dialysate sodium acetate, 0·10 *M* + acetic acid, 0·0105 *M*.
Nos. 540*, 541*, 542* methaemoglobin. All other experiments CO-haemoglobin.

No.	C	C_v	p	π_v	π_p	E observed	E calculated
533	0·58	0·59	1·89	3·25	3·21	—	+0·05
534	0·95	0·96	3·01	3·15	3·13	—	+0·09
514	1·44	1·46	4·80	3·30	3·27	—	+0·13
513	1·86	1·90	6·52	3·44	3·39	+0·10	+0·17
512	2·72	2·79	9·36	3·36	3·29	+0·28	+0·25
511	5·46	5·62	21·2	3·77	3·63	+0·63	+0·50
510	8·07	8·76	33·7	3·85	3·63	—	+0·80
509	12·34	14·00	63·4	4·53	4·18	+0·90	+1·25
535	12·97	14·82	67·5	4·55	4·19	+1·33	+1·32
515	15·38	18·05	92·8	5·14	4·69	+1·70	+1·61
508	17·88	21·60	120·2	5·56	5·03	+1·75	+1·93
532	20·90	26·20	161·9	6·18	5·53	+2·67	+2·34
540*	3·30	3·41	11·36	3·33	3·19	+0·32	+0·41
542*	3·39	3·50	12·10	3·46	3·31	+0·47	+0·42
541*	3·77	3·91	13·39	3·42	3·25	+0·47	+0·47

V. The osmotic pressures of native globin and paraglobin of the horse.

In order to compare the osmotic pressures of globins prepared from crystallised and from uncrystallised haemoglobins, experiments were carried out on the globin of the horse, the haemoglobin of which can be crystallised without difficulty.

In the preparation 8 *b*, referred to below, crystals of the carbon monoxide haemoglobin of the horse were obtained by the addition at 0° of an equal volume of saturated ammonium sulphate to a solution of haemoglobin which had been separated from the stroma by the addition of ether and salt. The mother-liquor was removed on a Büchner filter, and the crystals were redissolved and recrystallised and freed from impurities by dialysis.

Preparations of globin were made by the method of Anson and Mirsky from this material and from uncrystallised haemoglobin. Unfortunately, the globin of the horse appears to be less stable than that of the ox, and most of the protein precipitated during the removal of ammonium sulphate by dialysis. Attempts to measure the osmotic pressure at p_H 7·7 were moreover unsuccessful, as most of the protein precipitated. Six determinations obtained at p_H 5·64 are recorded in Table VII.

The solutions were so dilute that the difference between the concentration C and the corrected concentration C_v is within the limits of experimental error. The calculation of the protein concentration from nitrogen determinations is not recorded, because the amounts of material available were insufficient for adequate analyses, and provisionally all the calculations have been based on refractometric measurements, assuming that the specific refraction increment for horse-globin is 0·00183, the same as that determined for ox-globin (ref.

Table VII. *Native globin and paraglobin of the horse, osmotic pressures at* p_H *5·6 and 0°.*

Preparation 8 *a*, native globin from uncrystallised haemoglobin; preparation 8 *b*, native globin from recrystallised haemoglobin; preparation 8 *c* paraglobin.

Preparation No.	Exp. No.	C	p	E	π^*	Test for paraglobin
8 *b*	497	0·68	3·94	+0·23	5·8	Trace
8 *b*	498	0·68	3·85	+0·36	5·7	Some
8 *b*	499	0·45	2·80	—	6·2	Trace
8 *b*	500	0·47	2·66	+0·28	5·7	Some
8 *a*	501	1·11	6·43	+0·50	5·8	Much
8 *a*	502	0·60	3·63	—	6·1	Trace
8 *c*	505	1·89	5·00	+0·85	2·7	—
8 *c*	506	0·94	2·54	—	2·7	—
8 *c*	507	0·95	2·72	—	2·9	—

$\pi^* = p/C$.

Section VI). In the solutions containing less than 0·5 g. of protein per 100 cc., the error in the refractometric determinations may be about 5 %.

It appears that the mean value for π is 5·88, whereas the value obtained for ox-globin is about 4·58. The difference is about four times as great as the maximum observed deviation from the mean value 5·88, and it seems probable that the difference is a real phenomenon. In the second place, it appears that the osmotic pressures of the globins prepared from recrystallised and from uncrystallised haemoglobin are the same, within the limits of experimental error. As in the case of ox-globins, the osmotic pressure of the paraglobin of the horse is less than half the pressure given by the native globin.

VI. A comparison of the densities and the specific refraction increments of globin and haemoglobin.

In a number of the experiments made in this research, the measurements of osmotic pressure were supplemented by determinations of the specific refraction increments and the densities of the protein solutions.

The measurements of densities are recorded in Table VIII. It seems that for both proteins, the density of the protein solution, D', is directly proportional to the concentration C expressed in g. per 100 cc. of solution, as stated in formula (4).

$$D' = D'' + Ck_d \qquad \text{.....(4).}$$

D'' = density of the dialysate; k_d = an empirical coefficient. This formula has been used in calculations of osmotic pressures of dilute solutions, in which the observed heights of the columns in cm. of protein solution must be reduced to mm. mercury. The values of k_d have been calculated to five places, but the fifth figure is uncertain, and for the purposes of this work it is sufficient to determine k_d approximately to four places. The values of k_d for haemoglobin and for globin in both the solvents used are 0·00256 $\pm$ 0·0001. It is not yet possible to say whether the slight deviations from 0·00256 are of any significance.

Table VIII. *Densities of solutions of globin and haemoglobin of ox, equilibrated with buffer mixtures at 0°.*

Buffer 7·7. Na_2HPO_4, 0·03214 M + KH_2PO_4, 0·00357 M.
Buffer 5·6. CH_3COONa, 0·10 M + CH_3COOH, 0·0105 M.

No.	Protein	p_H	Density of dialysate D''	Density of protein D'	C	k_d
544	CO-haemoglobin	7·7	1·0047	1·0404	14·26	0·00250
543	,,	7·7	1·0050	1·0467	16·51	0·00253
515	,,	5·6	1·0045	1·0435	15·41	0·00253
508	,,	5·6	1·0045	1·0496	17·89	0·00252
532	,,	5·6	1·0046	1·0561	20·90	0·00247
540	Methaemoglobin	5·6	1·0043	1·0126	3·30	0·00250
527	Globin	7·7	1·0051	1·0093	1·58	0·00266
518	,,	7·7	1·0046	1·0119	2·77	0·00263
516	,,	7·7	1·0053	1·0152	3·95	0·00252
525	,,	5·6	1·0045	1·0108	2·49	0·00250
488	,,	5·6	1·0041	1·0238	7·54	0·00261

Measurements of the specific refraction increments of haemoglobin and of globin are recorded in Table IX. The numbers of the experiments have been stated and other data concerning the protein concentration and conditions in these experiments are given in Tables I, II, III and VI. In accordance with previous work on the serum-proteins [Adair and Robinson, 1930, 1] it was found that the value of α', defined by formula (5), is independent of the protein concentration, C, expressed in g. per 100 cc. of solution.

$$R' - R'' = \alpha' C \qquad \text{.....(5).}$$

R' = refractive index of protein solution; R'' = refractive index of dialysate. All dialyses were carried out at 0° but the measurements of R' and R'' were made at about 15°.

Table IX. *Specific refraction increments of carbon monoxide haemoglobin and of globin of the ox, equilibrated with phosphate and acetate buffers at 0°.*

Haemoglobin			Globin p_H 7·7		Globin p_H 5·6	
Exp. No	Buffer	α'	Exp. No.	α'	Exp. No.	α'
543	7·7	0·001955	516	0·00180	481	0·00183
544	7·7	0·001935	526	0·00179	486	0·00182
545	7·7	0·001965	527	0·00179	488	0·00185
546	7·7	0·001941	528	0·00176	489	0·00182
508	5·6	0·001955	530	0·00175	525	0·00181
513	5·6	0·001988	536	0·00178	531	0·00194
515	5·6	0·001960	539	0·00178	538	0·00189
534	5·6	0·001960	—	—	—	—

Theoretically, there may be appreciable differences between α' and α, the specific refraction increment of the protein in water, because $R' - R''$ is affected by the unequal distribution of salts across the membrane. The refraction increments of the salts in the dialysates were estimated by deducting the refractive index of water, and the results ranged from 0·0011 to 0·0012 for the acetate buffer at p_H 5·6 and from 0·00095 to 0·00099 for the phosphate buffer at p_H 7·7. In the case of globin at p_H 5·6, the membrane potentials

indicate that there is an appreciable excess of acetate ions inside the membrane, and it is possible that the refraction due to the diffusible ions is greater than 0·0012. In the systems containing haemoglobin and globin at p_H 7·7 the potentials are small, and it is unlikely that there is much difference between the refractions due to salts on both sides of the membrane.

In the case of human oxyhaemoglobin, α is 0·001942 [Stoddard and Adair, 1923], and we have obtained a similar value of α, namely 0·001952 for the haemoglobin of the ox. The values of α' recorded in Table IX agree with 0·00195 within the limits of experimental error.

In the case of the native globin of the ox at p_H 7·7, the mean value of α' is 0·00178. The earlier experiments on native globin at p_H 5·6 gave a mean value for α' of approximately 0·00183, but some of the experiments equilibrated for longer periods gave higher values and in the case of unfractionated globin (preparation 4, Table III) the following figures were obtained: 0·00190, 0·00188, 0·00188, 0·00190. It seems possible that the higher values are due to the presence of paraglobin, but the problem requires further investigation.

The values of α' for native globin at p_H 7·7 are similar to those obtained for egg-albumin and serum-albumin. Haurowitz and Waelsch [1929] have published lower values for globin, ranging from 0·00146 to 0·00154, but from the account of their experiment it seems unlikely that the concentration term C in their paper is the same as C in formula (5), which is calculated from nitrogen determinations, assuming that anhydrous globin contains 17·06 % of nitrogen. It is not improbable that the powdered globin used by Haurowitz and Waelsch was a hydrate containing less than 17 % of nitrogen. Schulz's [1898] preparations of globin, dried at a higher temperature (100°) contained about 16·89 % of nitrogen. Robertson [1912] prepared globin by Schulz's method and obtained values for α of 0·00169 $\pm$ 0·00005 for his purest preparation.

VII. Discussion.

In the formation of globin from haemoglobin by the action of acid acetone, 1 g. mol. or 67,000 g. of haemoglobin yields 4 g. mols or about 2525 g. haematin ($C_{34}H_{30}O_4N_4FeOH$) and the difference of about 64,475 must represent the mass of globin. The ideal osmotic pressure of a substance of this molecular weight is given by the formula $p/C_v = 2{\cdot}64$ at 0°.

The observed pressure ratios differ from 2·64. In discussing these differences, there are three factors which must be considered. These factors are represented by the symbols p_i, g_p and M in the general formula for the osmotic pressure of a protein, restated below.

$$p = p_i + p_p = p_i + RTg_p.10C_v/M \qquad \text{.....(6).}$$

p = observed osmotic pressure; p_i = ion pressure difference; p_p = partial osmotic pressure of protein ions; RT, the product of the pressure and volume of an ideal gas, = 22·4 × 760 = 17,033 at 0°; g_p = the osmotic coefficient of the protein ions, which represents the effects of attractive and repulsive forces

causing deviations from the ideal solution laws, M = the molecular weight, or the mean molecular weight if the protein is a mixture of aggregates. M may be a variable affected by the protein concentration if the aggregates can dissociate into smaller particles in accordance with the law of mass action.

The factor p_i can be calculated from membrane potential measurements, as described in Section IV, and the number of variables reduced to 2 by the calculation of the ratio π_p defined below.

$$\pi_p = p_p/C_p = 10RTg_p/M \qquad \text{.....(7).}$$

The results of calculations of π_p for the native globin of the ox are recorded in Fig. 2, in which π_p is plotted against C_p.

For comparison with the experiments on globin, a number of determinations of the osmotic pressure of haemoglobin in the same solvents, at p_H 7·7 and at p_H 5·6, have been made using carbon monoxide haemoglobin and methaemoglobin prepared from the stock solution of ox-haemoglobin utilised in the preparation of globins numbered 9 B to 9 K.

The osmotic pressures of CO-haemoglobin at p_H 7·7 may be considered first. Fig. 2 shows that π_v is about 2·6 in dilute solutions, and it increases as the protein concentration is increased. In both respects, this curve resembles the results obtained for the haemoglobins of the horse, the sheep, and of man, in solvents with p_H values ranging from 6·8 to 8·0, and with salt concentrations ranging from 0·01 to 0·23 mols per litre, recorded in previous papers [Adair, 1924; 1928].

It is almost certain that in the systems referred to above, the molecular weights of the proteins are constant, and that the changes in π_v are due to variations in the osmotic coefficients. If this condition is fulfilled it is legitimate to extrapolate the curve to determine the value of π_v when C_v is 0, as described by Adair and Robinson [1930, 2], and from the result obtained, namely 2·55 mm., it appears that the molecular weight of haemoglobin is about 67,000 in the case of the haemoglobin of the horse, sheep and man and the preparation of ox-haemoglobin used in this work.

The measurements on CO-haemoglobin and on methaemoglobin at p_H 5·6 show that π_v increases with increasing protein concentrations, an observation which proves that g_p must increase under these conditions, for any changes in aggregation which may occur tend to diminish π_p. In addition, the measurements indicate that π_p approximates to 3·15 in dilute solutions. Theoretically an effect of this order could be obtained if a certain proportion, not exceeding about 24 %, of the molecules dissociate into smaller units at p_H 5·6[1]. Svedberg and Nichols [1927] have shown that at p_H 5·4, the diffusion constant and the sedimentation velocity of haemoglobin differ by about 20 % from the normal

[1] Spectroscopic observations, for help with which we are greatly indebted to Prof. D. Keilin, revealed no denatured derivatives of haemoglobin in the material equilibrated for about 2 weeks at p_H 5·6 (in experiments numbered 540–542) but traces could be detected in material stored for longer periods.

values; their measurements being made on the CO-haemoglobin of the horse, at about 30°.

The results recorded in Fig. 2 show that there are remarkable differences between the osmotic pressures of globin and of haemoglobin. The osmotic pressure of globin is much more sensitive to changes in p_H, and the ratio π_p diminishes as the protein concentration is increased, even in the isoelectric region where π_p is equal to π_v.

Theoretically, the diminution in π_p must be due to a diminution of the osmotic coefficient, or a polymerisation of the protein, or to both of these factors acting in the same direction. There are two arguments in favour of the hypothesis that changes in polymerisation play an important part in the diminution of π_p. Firstly, this hypothesis agrees with the observation that, in dilute solutions, π_p at p_H 5·6 is two or three times as great as at p_H 7·7, for it is not improbable that an increase in acidity should favour the dissociation of the larger particles. Secondly, it appears to be a general rule that in the region of the isoelectric point, the osmotic coefficients increase rather than diminish. Since no exceptions to this rule have been found in previous investigations on the osmotic coefficients of the proteins, it seems reasonable to attribute the fall in π_p at the isoelectric point to changes in polymerisation rather than to a fall in the osmotic coefficient.

The arguments in favour of changes in the osmotic coefficient or of aggregation are more evenly balanced in the case of the ionised globin at p_H 5·6. The measurements of membrane potentials show that globin is more highly charged than haemoglobin, and according to the inter-ionic attraction theory of Debye and Hückel [1923] the osmotic coefficient should be diminished by an increase in the concentration of protein ions of high valency. On the other hand, the fact that the osmotic coefficient of haemoglobin in the same solvent fails to diminish is evidence in favour of the aggregation hypothesis. Nevertheless, as globin is more highly charged than haemoglobin at p_H 5·6 the diminution in π_p cannot be regarded as a definite proof of aggregation changes.

The calculation of the molecular weight of globin is complicated by the possibility that the protein undergoes polymerisation. The methods for the elimination of the effects due to the osmotic coefficient by extrapolation [Adair and Robinson, 1930, 2] cannot be applied to a system in which both M and g_p vary with the protein concentration and it will be seen that no attempt has been made to extrapolate the values of π_p for globin in Fig. 2. Although exact values cannot be obtained, rough estimates of the value of M for globin may throw light on the changes in aggregation which take place, and it is probable, though not certain, that in some of the systems studied in this work, the "uncorrected molecular weight" M/g, calculated by formula (7), is not greatly different from the corrected value. The results for native ox-globin at p_H 7·7 are given in Table X.

If M be a function of the protein concentration, the globin must be a mixture of polymerides. Svedberg and his colleagues have shown that there

Table X. *Effect of protein concentration on the uncorrected value of M for ox-globin at* p_H *7·7. (Subject to corrections of unknown magnitude.)*

(g. per 100 cc. solvent)	1·0	2·0	3·0	4·0	5·0	6·0
M (uncorrected)	94,000	110,000	123,000	139,000	150,000	155,000

are mixtures of aggregates in certain proteins, including their preparations of eu- and pseudo-globulins [Svedberg and Sjøgren, 1930; Mutzenbecher, 1931]. Svedberg [1930] has included globin in a list of proteins which are polydisperse. It is not improbable that the variations demonstrated by the centrifugal method are mainly due to the existence of simple physical mixtures of aggregates. Variations in *M* demonstrated by osmotic methods cannot be attributed to the existence of a simple physical mixture, and therefore it seems probable that in solutions of globin there is a dynamic equilibrium between the larger polymerides and the products of their dissociation.

The interpretation of the results obtained at p_H 5·6 presents greater difficulties. The measurements on native globins of the ox and the horse show that the ratio π_v is practically independent of the protein concentration over a considerable range. Since π_v and π_p approximate in dilute solutions, some provisional calculations of the value of *M* in dilute solutions have been made by using the observed values of π_v for comparisons with the results obtained for paraglobin.

Table XI. *Comparison of uncorrected molecular weights of different globins and paraglobins at* p_H *5·6 and approximately the same protein concentration (about 1 %).*

Ox native globin	37,000
Horse native globin	29,000
Ox paraglobin	99,000
Horse paraglobin	63,000
Ox unfractionated globin	52,000
Sheep unfractionated globin	125,000

The values of *M* for native globin are about one-half of the molecular weight of haemoglobin, but no conclusions can be drawn from this observation because *M* may vary with the protein concentration. It is clear that the degree of polymerisation is lower in native globin than in paraglobin or in unfractionated globin, and it may be that there is some correlation between the tendency for paraglobin to form larger aggregates and the fact that it can be precipitated more easily than native globin.

Summary.

The stability of globin at 0° in solutions buffered by phosphates and acetates has been investigated. In general globin is much less stable than haemoglobin, but preparations of the native globin of the ox have been made by the methods of Hamsik and of Anson and Mirsky, which give reproducible results in osmotic pressure measurements. The globin of the horse appears to be less stable than that of the ox.

In the case of ox-globin, the relationship between osmotic pressure and protein concentration has been studied at the isoelectric point, p_H 7·7, and at p_H 5·6, and for comparison with globin, experiments have been made on ox-haemoglobin, equilibrated with the same buffer mixtures. The partial pressure of the protein and the pressure due to the unequal distribution of ions have been estimated from measurements of membrane potential.

There is a striking difference between results obtained from globin and haemoglobin. In the case of globin, the ratio of partial pressure of protein and its concentration, π_v, tends to diminish as the protein concentration is increased. It is unlikely that this decrease is wholly due to deviations from the ideal solution laws, particularly in the case of isoelectric globin, and it seems probable that there is a dynamic equilibrium between polymerides of different sizes in solutions of globin, so that the average molecular weight is a function of the concentration of the protein, of hydrogen ion concentration and of other variables. At p_H 7·7, the mean molecular weight may be greater than that of haemoglobin.

Comparison of measurements of the osmotic pressure of unfractionated globin and of paraglobin with that of native globin at the same hydrogen ion concentration (p_H 5·6) indicates that paraglobin is more highly aggregated than native globin.

Preliminary investigations have been made on the osmotic pressures of the globins of the horse and the sheep.

REFERENCES.

Adair (1924). *Proc. Camb. Phil. Soc.* (Biol.), **1**, 75.
—— (1925). *Proc. Roy. Soc. Lond.* A **108**, 627.
—— (1928). *Proc. Roy. Soc. Lond.* A **120**, 573.
—— (1929). *Proc. Roy. Soc. Lond.* A **126**, 16.
—— Cordero and Shen (1929). *J. Physiol.* **67**, 288.
—— and Robinson (1930, 1). *Biochem. J.* **24**, 993.
—— —— (1930, 2). *Biochem. J.* **24**, 1864.
—— —— (1931). *J. Physiol.* **72**, 2 P.
Anson and Mirsky (1930). *J. Gen. Physiol.* **13**, 469.
Debye and Hückel (1923). *Physikal. Z.* **24**, 185.
Hamsik (1930). *Compt. Rend. Soc. Biol.* **104**, 243.
Haurowitz and Waelsch (1929). *Z. physiol. Chem.* **182**, 82.
Hill and Holden (1926). *Biochem. J.* **20**, 1326.
Hüfner (1887). Beitr. Physiol. (Carl Ludwig. Leipzig.)
Loeb (1922). Proteins and the theory of colloidal behaviour. (New York.)
Mutzenbecher (1931). *Biochem. Z.* **235**, 425.
Robertson (1912). *J. Biol. Chem.* **13**, 455.
Roche (1929). *Archiv. Physique Biol.* **7**, 165.
—— (1930). *Compt. Rend. Trav. Lab. Carlsberg*, **18**, No. 4.
Schulz (1898). *Z. physiol. Chem.* **24**, 449.
Stoddard and Adair (1923). *J. Biol. Chem.* **57**, 437.
Svedberg (1930). *Kolloid Z.* **51**, 10.
—— and Fåhreus (1926). *J. Amer. Chem. Soc.* **48**, 430.
—— and Nichols (1927). *J. Amer. Chem. Soc.* **49**, 2920.
—— and Sjøgren (1930). *J. Amer. Chem. Soc.* **52**, 2855.

CCXV. THE CHEMISTRY OF THE WHITE ROTS OF WOOD.

III. THE EFFECT ON WOOD SUBSTANCE OF *GANODERMA APPLANATUM* (PERS.) PAT., *FOMES FOMENTARIUS* (LINN.) FR., *POLYPORUS ADUSTUS* (WILLD.) FR., *PLEUROTUS OSTREATUS* (JACQ.) FR., *ARMILLARIA MELLEA* (VAHL.) FR., *TRAMETES PINI* (BROT.) FR., AND *POLYSTICTUS ABIETINUS* (DICKS.) FR.

By WILLIAM GEORGE CAMPBELL.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Bucks.

(*Received September 14th, 1932.*)

In two previous communications [Campbell, 1930; 1931] experimental data have been adduced to indicate that white rot fungi are in no sense uniform with respect to the order in which they attack the principal components of wood. In the present investigation a further series has been examined with a view to determining whether the white rots can be classified into groups on the basis of their chemical action—individual members which attack wood in the same manner being included in the same group.

Experimental.

The experimental procedure and analytical methods were similar in all respects to those used in previous work except in the case of the decay caused by *A. mellea* which will be discussed later. The fungi to be studied were divided into three groups as follows:

1. *G. applanatum*, *F. fomentarius*, *P. adustus* and *P. ostreatus*.

The material to be decayed by this group of fungi consisted of a well-mixed sample of fresh, air-dried 60–80 mesh beech wood flour. Prior to inoculation each portion of wood flour was submitted to the usual sterilisation treatment, and the decayed wood was analysed after washing free from acid with cold water and drying at 105°.

The effect of 1 % NaOH at 100° was determined as before on both sound and decayed wood. The analytical results which are expressed as percentages by weight of the original oven-dry wood are recorded in Table I.

Table I. *The effect of a series of white rots on the chemical composition of beech wood, together with the effect of decay on the alkali-solubility of the residual wood substance.*

(Results expressed as percentages by weight of original oven-dry wood.)

		Decayed by:			
	Original wood	*Ganoderma applanatum*	*Fomes fomentarius*	*Polyporus adustus*	*Pleurotus ostreatus*
Before extraction with 1 % NaOH					
Duration of decay	—	2 months	4 months	4 months	4 months
Loss due to decay *plus* cold water washing	—	10·61	15·75	10·50	8·79
Cold water-soluble	1·03	1·32	1·03	0·89	0·45
Hot water-soluble	1·49	2·65	2·40	2·33	1·29
1 % NaOH-soluble	—	—	—	—	—
Cellulose	59·52	55·34	50·70	53·08	55·29
Lignin	20·54	16·81	16·00	18·03	18·60
Methoxyl	5·82	5·03	4·67	5·02	4·41
Total pentosans	22·28	19·46	18·40	19·76	19·86
Pentosans in cellulose	12·65	11·93	9·94	10·60	11·36
Pentosans not in cellulose	9·63	7·53	8·46	9·16	8·50
After extraction with 1 % NaOH for 1 hour at 100°					
1 % NaOH-soluble	14·57	19·73	18·33	18·75	17·00
Cellulose	58·49	47·12	46·9	50·52	49·99
Lignin	18·96	13·04	12·14	13·26	16·01
Methoxyl	4·66	3·46	3·44	3·71	2·39
Total pentosans	17·07	14·15	12·07	14·28	14·92
Pentosans in cellulose	11·75	7·83	8·51	10·04	9·42
Pentosans not in cellulose	5·32	6·32	3·56	4·24	5·50

In the samples inoculated with *G. applanatum* and *F. fomentarius* moderately large, tough wefts of mycelium formed on the surface of the decaying wood. In each case the weft was removed in a single sheet, carefully washed free from adhering particles of wood and weighed separately after drying at 105°. From 130·32 g. (oven-dry) of original wood decayed by *G. applanatum* 2·76 g. or 2·12 % of mycelium was obtained, while in the case of *F. fomentarius* 3·93 g. or 3·02 % was obtained from the same weight of wood. In the case of *P. adustus* and *P. ostreatus* the surface growth of mycelium was almost negligible in amount.

2. Heart rot of cherry wood (*Prunus avium* L.) caused by *Armillaria mellea*.

In a previous communication [Campbell, 1931] the effect of *A. mellea* on beech wood was described in some detail. At the stage of decay which was examined, however, the loss in weight was only 8·66 % and no attack on lignin was observed. Since these results were published, material in the form of an air-dry disc of cherry wood showing pronounced heart-rot caused by the same fungus has become available, and this has been examined with a view to gaining information regarding the more advanced stages of decay. In order to obtain an approximate measure of the loss in dry weight sustained by the central portion of the disc during decay it was necessary in the first instance to

determine the specific gravity of both sound and decayed wood. Eight specimens measuring approximately 1·5 × 1·5 × 5 cm. were cut at random from the outer sound heartwood of the disc and four samples were taken from the relatively small decayed central portion. It was considered advisable to determine the volumes of the specimens without wetting them and the author is indebted to Mr E. D. van Rest for drawing his attention to a paper by Hartmann, Westmont and Morgan [1926] from which it was possible to evolve a simple method of determining the volumes of wood samples by means of the displacement of sand. In the paper referred to it is stated that the bulk volume of a specimen of any porous material may be calculated from the difference in volume of sand displaced by the specimen and a metallic standard of approximately the same size and shape. It is shown that when this difference is small it is possible to determine the volume of the specimen with the same degree of precision as it is possible to measure the metallic standard. The fine sand employed in the present experiments was freed from organic matter, sifted to obtain 80–100 mesh material thoroughly washed and dried. The procedure employed was essentially the same as that indicated by Hartmann, Westmont and Morgan [1926] except that the operations were carried out on a much smaller scale. The sand was held in a filter-funnel to the lower end of which was fitted a short length of rubber tubing provided with a spring clip. A small cylindrical glass vessel served to retain the wood specimens and the steel standard, which latter measured 1·48 × 1·48 × 5 cm. at 15°.

It was found possible, with practice, to duplicate the weight of annular sand round either the standard or test pieces to within 0·2 g. in the majority of cases. With sand of bulk sp. gr. 1·455, 0·2 g. corresponds to a volume of 0·137 cc., so that, by accepting in each case the average of the two most closely approximating weights of annular sand it was considered that this method of determining the volumes of test pieces gave sufficiently accurate results.

After the air-dry volumes had been obtained the air-dry and oven-dry weights of each sample were determined. The results of the specific gravity determinations are given in Table II.

After conversion into wood flour of 60–80 mesh the sound and decayed materials were analysed in the usual manner (Table III). In view of the slight divergences in the moisture contents of the sound and decayed samples (Table II), the approximate loss in weight sustained by the wood which was decayed cannot be directly assessed by means of the true specific gravity figures but rather by comparing the averages of the quotients obtained in both series by dividing the dry weight of each sample by its air-dry volume. It can then be assumed, by neglecting the probable effect of partial decay on the swelling properties of wood and the normal slight variation in specific gravity found in all woods in passing from the centre of the tree outwards, that at any given temperature and relative humidity a given volume of the sound cherry heartwood contains more wood substance than the same volume of the

Table II. *Comparison of specific gravity of sound cherry heartwood with that of cherry heartwood of the same tree decayed by* A. mellea.

Specimen No.	Volume in cc. (air-dry)	Weight in g. (air-dry)	Weight in g. (oven-dry)	Moisture content % of oven-dry weight	Specific gravity Wt. (air-dry) / Vol. (air-dry)	Wt. (oven-dry) / Vol. (oven-dry)
			Sound wood			
1	10·657	7·361	6·555	12·3	0·691	0·615
2	6·038	4·528	4·029	12·46	0·750	0·667
3	8·873	5·469	4·862	12·46	0·616	0·548
4	10·149	6·945	6·185	12·28	0·684	0·609
5	11·867	7·448	6·675	11·59	0·628	0·562
6	10·763	7·042	6·304	11·71	0·654	0·586
7	11·647	7·718	6·897	11·91	0·663	0·592
8	9·49	6·519	5·812	12·17	0·687	0·612
				Average	0·672	0·599
			Decayed wood			
1	12·409	5·735	5·059	13·38	0·462	0·408
2	14·583	7·743	6·819	13·21	0·531	0·468
3	8·763	5·278	4·654	13·38	0·602	0·531
4	7·513	3·228	2·843	13·55	0·430	0·378
				Average	0·506	0·446

decayed wood in the proportion of approximately 1·34 : 1, or, alternatively that during decay the central portion of the disc has lost approximately 25 % of its oven-dry weight. By employing this latter figure the analytical data for the decayed wood can be expressed as percentages of the oven-dry weight of the original wood (Table III).

Table III. *The effect of heart-rot caused by* A. mellea *on the wood of cherry* (Prunus avium *L.*).

	Original wood %	Decayed wood %	Decayed wood % sound wood
Loss in weight due to decay	—	—	25·00 (approx.)
Cold water-soluble	2·97	4·47	3·35
Hot water-soluble	6·49	7·21	5·41
1 % NaOH-soluble	26·44	29·15	21·86
Cellulose	53·74	53·74	40·30
Lignin	19·32	20·97	15·73
Total pentosans	25·75	27·59	20·69
Pentosans in cellulose	13·20	14·31	10·73
Pentosans not in cellulose	12·55	13·28	9·96

3. *Trametes pini* and *Polystictus abietinus.*

The effect of *T. pini* was observed on a sample of silver fir (Table IV). In this case insufficient material was available for a detailed examination of the effect of 1 % NaOH on the sound and decayed wood. In the case of the decay caused by *P. abietinus* the starting material was 60–80 mesh Norway spruce and in this case both decayed and sound wood were analysed before and after extraction with 1 % NaOH for 1 hour (Table V).

Table IV. *Analysis of 60–80 mesh silver fir wood before and after decay by* Trametes pini.

Results expressed as percentages by weight of original oven-dry wood.

	Original wood	Decayed wood
Duration of decay	—	17 months
Loss due to decay *plus* cold water washing	23·01	—
Cold water-soluble	1·02	0·92
Hot water-soluble	1·87	2·5
1 % NaOH-soluble	12·0	12·98
Cellulose	57·46	46·47
Lignin	29·35	22·87
Methoxyl	4·81	3·7
Total pentosans	9·41	6·76
Pentosans in cellulose	3·18	2·61
Pentosans not in cellulose	6·23	4·15

Table V. *The effect of* Polystictus abietinus *on the chemical composition of the wood of Norway spruce, together with the effect of the decay on the alkali-solubility of the residual wood substance.*

Results expressed as percentages by weight of original oven-dry wood.

	Before extraction with 1 % NaOH		After extraction with 1 % NaOH for 1 hour at 100°	
	Original wood	Decayed wood	Original wood	Decayed wood
Duration of decay	—	5 months	—	—
Loss due to decay *plus* cold water washing	—	6·79	—	—
Cold water-soluble	1·67	1·64	—	—
Hot water-soluble	2·77	4·7	—	—
1 % NaOH-soluble	—	—	13·54	20·27
Cellulose	60·54	55·08	55·72	51·08
Lignin	26·22	23·16	23·85	17·36
Methoxyl	4·7	3·98	4·17	2·92
Total pentosans	10·28	8·87	8·59	6·22
Pentosans in cellulose	4·12	3·73	4·16	3·59
Pentosans not in cellulose	6·16	5·14	4·43	2·63

Discussion of results.

The results in Table I reveal that each fungus in the group has attacked carbohydrates as well as lignin. Well-defined differences with regard to the proportions of each constituent attacked, however, are at once apparent. For instance, *G. applanatum* and *P. adustus* acting on wood taken from the same original sample have, over widely different times, brought about an equal loss in weight and yet the latter has caused a greater depletion of cellulose than the former. This is in part compensated for by the fact that *G. applanatum* has a more pronounced action on lignin. Further differences are observed in the manner in which these two fungi attack the furfuraldehyde-yielding complexes of the wood. *G. applanatum* produces a relatively greater amount of depletion of the pentosans not associated with the cellulose than of the pentosans in the cellulose, whereas the reverse is true in the case of *P. adustus*. In the case of *G. applanatum* 2·12 parts of the 10·61 % loss in weight caused by the decay can be directly accounted for by the formation of a weft of mycelium

on the surface of the decaying wood while only a negligible amount of surface mycelial growth was observed on the wood decayed by *P. adustus*. These considerations point to pronounced differences in the biological activities of the two fungi although in a general sense they have the same ultimate chemical action on wood.

Comparison of the wood samples decayed by *P. adustus* and *P. ostreatus* respectively for the same length of time reveals that the two fungi deplete lignin to approximately the same extent although *P. ostreatus* causes a smaller total percentage loss of wood substance. At the same time at the stages of decay examined cellulose appears to be more resistant to *P. ostreatus* than to *P. adustus*. Slight differences in the respective effects of the two fungi on the furfuraldehyde-yielding complexes are also observed despite the fact that the depletion of total pentosans is the same in both cases.

In Table I the greatest degree of decomposition of the beech wood has been brought about by *F. fomentarius* but the net chemical effect only differs from that of *G. applanatum* in a few particulars. Much more cellulose has been decomposed while the extent of lignin depletion is not much more than in the decay caused by *G. applanatum*. The attack on pentosans is slightly more severe and the pentosans in the cellulose suffer proportionately greater depletion than the pentosans not associated with the cellulose. *F. fomentarius* resembles *G. applanatum* in that a considerable amount of mycelium is laid down, 3·02 parts of the 15·75 % loss in weight of the original wood being accounted for in this way.

Further examination of the analytical data reveals that each of the four fungi under consideration brings about an increase in the alkali-solubility of the residual wood substance at the stages of decay examined, but reference to a group of data recorded by Hawley and Wise [1926, p. 298] shows clearly that for equal percentage losses of wood substance all the white rots examined so far induce much smaller increases in alkali-solubility than the brown rots. It has been indicated previously [Campbell, 1930; 1931] that herein lies one of the principal chemical bases of differentiation between the two types of wood decay. In brown rots increase in alkali-solubility is roughly proportional to the amount of cellulose depletion and does not reach a maximum until a comparatively late stage of decay. In the white rots under consideration there is no proportionality between alkali-solubility and loss of cellulose or any other constituent.

The data in Table III illustrate the type of difficulty which is likely to arise in attempting to compare sound wood with wood which has been decayed by a white rot where the loss in weight due to decay is not known. It is suggested, however, that the method here used to determine loss in weight is sufficiently accurate for the purpose of the investigation, since it serves to confirm a previous conclusion [Campbell, 1931] that the decay caused by *A. mellea* must on purely chemical grounds be regarded as a variety of white rot. When the analytical data for the decayed wood are calculated as per-

centages by weight of oven-dry sound wood, it is observed that a stage has been reached at which lignin is decomposed, although the constituent which has been most severely attacked is undoubtedly the cellulose. As in the early stages of decay the pentosans associated with the cellulose have suffered depletion as well as the pentosans not in the cellulose. Finally, a definite decrease in alkali-solubility has taken place. At the stage under review the decay caused by *A. mellea* is closely similar in its effects to other white rots which have been studied.

The results in Tables IV and V illustrate the respective effects of two white rots on gymnospermic woods. *Trametes pini* has figured largely in the literature on wood decay as a typical white rot fungus in that it has been supposed to confine its attack to the lignin of wood and to leave the cellulose unchanged. The data in Table IV indicate clearly that no such specific action takes place and that the white rot caused by *T. pini* is comparable in general with other white rots which have been studied. The results appear to agree with the findings of Hawley, Wiertelak and Richards [1930] with regard to the same fungus.

Thaysen and Bunker [1927, p. 129] have quoted evidence in support of the view that *P. abietinus* causes a rot in which cellulose is depleted in the early stages but in which lignin is not attacked until decay is well advanced. The data in Table V, however, do not bear this out. At the early stage of decay under investigation, the attack on lignin is proportionately greater than the attack on cellulose, and of the two groups of pentosans those which are not associated with the cellulose are more susceptible to attack. In this case there is a comparatively sharp rise in alkali-solubility as cellulose and more especially lignin become more soluble in alkali as a direct result of attack. Unlike most of the other examples studied the pentosans not in the cellulose are more soluble in alkali than those which are associated with the cellulose.

Conclusions.

Sufficient data have now been obtained to show that as a class the white rot fungi are not consistent in the manner of their attack on wood, for although none has yet been found to have a specific action on lignin there is no general uniformity with regard to either the order or proportion in which the major wood components are decomposed. The evidence suggests that the white rots should be subdivided on chemical grounds into the following three groups.

Group I.

White rots in which lignin and pentosans are attacked in the early stages and in which the incidence of the attack on the cellulose proper is delayed. Representative fungus: *Polystictus versicolor* [Campbell, 1930; Lutz, 1931].

Group II.

White rots in which cellulose and its associated pentosans are attacked in the early stages and in which the incidence of the attack on lignin and pentosans not associated with the cellulose is delayed. Representative fungus: *Armillaria mellea* [Campbell, 1931].

Group III.

White rots in the early stages of which both lignin and cellulose are attacked but in varying proportions. Representative fungi: *Polyporus hispidus* [Campbell, 1931], *Ganoderma applanatum*, *Polyporus adustus*, *Pleurotus ostreatus*, and *Polystictus abietinus*.

It is considered that an early stage of decay might reasonably be regarded as one at which the observed loss in weight calculated as a percentage of the oven-dry original wood is approximately between 5 and 10. There is, therefore, insufficient evidence on which to classify *F. fomentarius* and *T. pini*, although the probability is that they will be found to fall into Group III. From an examination of the data obtained by Falck and Haag [1927] with respect to *Fomes annosus* it appears that this fungus also belongs to Group III, since if the specific gravity figures recorded by these authors be used in the manner previously indicated to calculate the results for the decayed wood as percentages of the weight of oven-dry sound wood it can be seen that the cellulose content as well as the lignin content decreases in the early stages of decay. In the same way it can be shown that the percentage alkali-solubility begins to decrease after the loss in weight of the original wood exceeds 20 to 25 % of the oven-dry original wood.

It should not be overlooked that the mere fact that it is possible to subdivide the white rots on the lines indicated above cannot be taken to indicate that each fungus induces a set of chemical reactions peculiar to the group to which it belongs. It would obviously be difficult at very advanced stages of decay to differentiate between one white rot and another by chemical means, and it is therefore highly probable that the ultimate primary reactions involved are the same in all three groups but that the main variants are the incidence in point of time of the primary reactions, the course of secondary reactions, if any, and the rates of the several reactions. It has been pointed out elsewhere [Campbell, 1932] that the principal enzymic reactions which are generally associated with the decay of wood by fungi are hydrolysis and oxidation. In brown rots hydrolysis appears to be the only primary reaction [Campbell and Booth, 1929] but it is suggested that in white rots decomposition is brought about by the combined action of oxidation and hydrolysis. In the present state of knowledge it appears that it is only on this basis that the observed behaviour of white rot fungi can be explained. The different rates of attack on the major wood components must be due in large measure to differences in the incidence and extent of secretion of at least two kinds of

enzymes with widely differing propensities. This contention derives support from the observation of Nutman [1929] that *Polyporus hispidus* secretes both hydrolysing and oxidising enzymes, while according to Lutz [1931] the same holds true for *Polystictus versicolor*, but, in addition, this fungus is capable of inducing at least one secondary reaction which results in the formation of alcohol from the products of the primary hydrolysis.

Consideration of the effect of white rot fungi on the pentosans of wood presents some difficulty. In brown rots, for instance, it is a general rule that of the two categories of pentosans those which are associated with the cellulose are the more susceptible to attack, but in white rots it appears that sometimes the one category and sometimes the other suffers relatively greater depletion without any reference to the extent of depletion in either the cellulose proper or lignin. In view of the above suggestion that both oxidising and hydrolysing enzymes play a part in all white rots it is not improbable that the true effect of decay on the wood pentosans is to some extent masked, for on the one hand it is known that oxycellulose yields a certain amount of pentose on hydrolysis, and on the other hand there is the possibility in some cases of the formation during decay of insoluble by-products which yield furfuraldehyde on hydrolysis with hydrochloric acid.

There appear to be good grounds for the conclusion that in white rots the solubility of the decayed wood in alkali rises to a maximum at a much earlier stage of decomposition than in brown rots. In certain instances [Campbell, 1930; 1931] this has been ascribed to the fact that the pentosans in decayed wood which are not associated with the cellulose are less soluble in alkali than the corresponding complexes in sound wood, but it is obvious from the results of the present investigation that this is not true for all white rots, since here it has only been found to hold for beech wood decayed by *G. applanatum* and *P. ostreatus*. These results appear to confirm the suggestion made in the preceding paragraph concerning the formation of by-products during decay. It is therefore obvious, in view of the general effect observed on the total alkali-solubility of wood decayed by white rot fungi, that the solubility of some major constituent other than pentosans must be consistently affected. It has been shown [Campbell and Booth, 1929] that in brown rots hydrolysis has the direct effect of increasing the alkali-solubility of cellulose, and Bray [1924] has shown that in the same type of decay the increase in the total alkali-solubility of the residual wood is proportional to the loss in cellulose. The suggestion accordingly presents itself that in white rots the full effect of hydrolysis on the alkali-solubility of cellulose is either masked or partially inhibited by the action of oxidases.

Summary.

1. A detailed chemical examination of a further series of white rots of wood tends to strengthen a previous conclusion that fungi of the white rot type invariably decompose carbohydrates as well as lignin, although there is

no general uniformity with regard to either the order or proportion in which the several wood components are decomposed.

2. The white rots can be subdivided on chemical grounds into three distinct groups as follows.

(i) White rots in which lignin and pentosans are attacked in the early stages and in which the incidence of the attack on cellulose is delayed.

(ii) White rots in which cellulose with its associated pentosans is attacked in the early stages and in which the incidence of the attack on lignin and the pentosans not in the cellulose is delayed.

(iii) White rots in the early stages of which both lignin and cellulose are attacked but in varying proportions.

3. It is suggested that the ultimate primary reactions involved in all white rots are oxidation and hydrolysis and that the main variants on which the above subdivision depends are the incidence in point of time of the primary reactions, the course of secondary reactions if any, and the rates of the several reactions.

4. In white rots the total alkali-solubility of the decayed wood as a percentage of oven-dry sound wood rises to a maximum and begins to decline at a much earlier stage than in brown rots.

The author desires to express his indebtedness to Messrs K. St G. Cartwright and W. P. K. Findlay for carrying out the inoculations, to Mr D. F. Packman for assistance in the Laboratory work, to Sir James Irvine for criticising the manuscript and to Mr R. S. Pearson, Director of Forest Products Research, for permission to publish these results.

REFERENCES.

Bray (1924). *Paper Trade J.* **78** (23), 58.
Campbell (1930). *Biochem. J.* **24**, 1235.
—— (1931). *Biochem. J.* **25**, 2023.
—— (1932). *Forestry*, **6**, 82.
—— and Booth (1929). *Biochem. J.* **23**, 566.
Falck and Haag (1927). *Ber. deutsch. chem. Ges.* **60**, 225.
Hartmann, Westmont and Morgan (1926). *J. Amer. Ceramic Soc.* **9**, 298.
Hawley and Wise (1926). Chemistry of wood. (Chemical Catalog Co.)
—— Wiertelak and Richards (1930). *Cellulosechemie*, **11**, 259.
Lutz (1931). *Bull. Soc. Chim. Biol.* **23**, 436.
Nutman (1929). *Ann. Appl. Biol.* **26**, 40.
Thaysen and Bunker (1927). Microbiology of cellulose, hemicelluloses, pectin and gums. (Humphrey Milford.)

CCXVI. A NOTE ON MOVEMENTS AND PRECIPITATIONS OF AN AMPHOTERIC COLLOID DUE TO AN ELECTRIC FIELD AND SLIGHT ELECTROLYSIS.

A MODIFIED HARDY'S EXPERIMENT SUITABLE FOR DEMONSTRATION PURPOSES.

By WILLIAM JAMES PENFOLD and JAMES SUTHERLAND.

From the Baker Institute of Medical Research, Alfred Hospital, Melbourne.

(*Received September 16th, 1932.*)

While observing microscopically the cataphoretic movement of a gelatin-protected silver colloid it was found:

(1) That the particles in the centre of the field migrated towards the anode.

(2) That a wave of particles emerged from each electrode.

(3) That these waves ultimately met near the centre of the field, where they precipitated.

Hardy [1899] described similar effects using, however, U-tubes and boiled egg-white solution. Our experiment presents the same phenomena, but is so simply performed as to be suitable for class work.

It is conducted in large cells, the walls of which are formed by two glass plates (selected flat lantern slides do well for this purpose). The individual particles, of course, cannot be observed by this method, but the essential facts relative to the electrical phenomena known to occur in amphoteric colloids can be observed or deduced.

The nature of the silver colloid.

The silver colloid we employed was prepared by Bredig's method in a menstruum of 0·5 % gelatin (Coignet's best) in water. No salts were added separately. A slight amount of electrolysis occurs in the experiment. After 10–20 minutes' sparking with about 5 amp. passing at 230 v. d.c., the fluid acquires a dark brown colour, due to the colloidal dispersion of the silver. The colloid so obtained should be centrifuged to remove the larger particles, after which it may be preserved for months if it be protected from the influence of light. On examination under the high power microscope, using dark ground

illumination and a red light, innumerable minute particles are seen. The particles are, of course, in active Brownian movement and are mostly smaller than 0·2 $m\mu$ in diameter. As before mentioned, these particles migrate towards the anode in an electric field, showing that they are negatively charged. The reaction of the colloid is slightly alkaline.

During the preparation of the colloid, in the manner described, each particle of silver is considered to acquire an envelope of gelatin. This envelope apparently imparts properties to the colloid which are essentially those of gelatin. From this point of view the phenomena of the experiment will be discussed. The silver nucleus acts in the macroscopic experiment as a visible indicator of the location of the particles.

Experimental.

The two lantern slides must be chemically clean. For this purpose they are treated with chromic acid, water and alcohol, the alcohol being removed by burning rather than wiping. The glass plates are kept 0·03 mm. apart by the insertion of platinum foil electrodes at the opposite sides of the cell. The cell is completely filled with the colloidal solution, care being taken to exclude all air bubbles. Excessive colloid is wiped away from the edges of the cell before sealing it with melted paraffin. The paraffin is applied by means of a hot glass or metal rod. The finished cell will have a light yellowish-brown colour. The electrodes are then connected to a source of electricity, with the results shown in the photographs (Plate VII, Figs. 1 to 5). These were taken at the times indicated without interrupting the experiment, panchromatic plates being used. The cell was placed on a glass platform and illuminated from below, the camera being placed overhead in the inverted position.

The points to be noted are the following.

(1) A wave of particles first appears emerging from the negative electrode soon after the current is applied. This indicates that the particles of the colloid have a negative charge. The photographs demonstrate that the wave which comes from the negative electrode is smooth and shows no signs of precipitation.

(2) After a short but perceptible lag a much denser wave is seen to emerge from the positive electrode. The lag in the appearance of the positive wave is due to the attraction exerted by the positive electrode on the negatively charged particles of the colloid. No repulsion of these particles by the positive electrode can occur until their charge has changed its sign. This is effected by the electrolytic production of acid at the positive electrode. As soon as this change of sign has occurred the particles tend to be repelled from the electrode and from each other. In the process of changing the sign of the charge, precipitation of the particles occurs and this precipitation accounts for the greater density of the positive wave. Moreover, this wave shows an

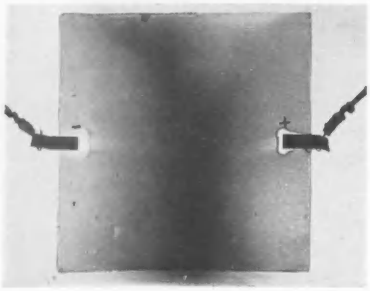


Fig. 1.

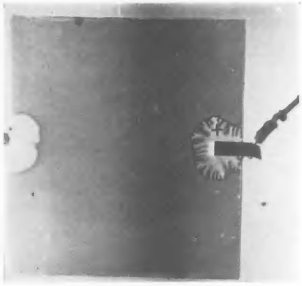


Fig. 2.

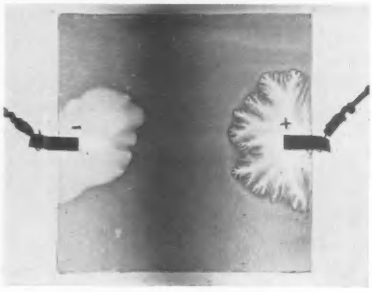


Fig. 3.

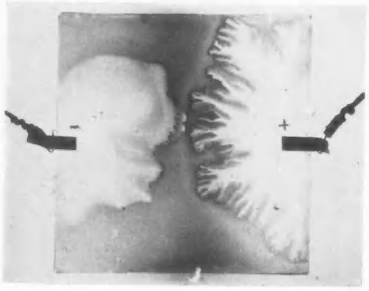


Fig. 4.

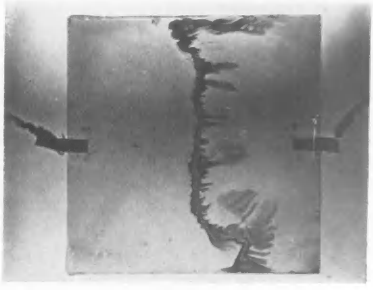


Fig. 5.

Figs. 1–5. Photographs of macroscopic experiment in progressive stages.

Fig. 1. Taken 3 minutes after application of 20 volts D.C.
Fig. 2. Taken after 10 minutes from beginning of experiment.
Fig. 3. Taken after 50 minutes from beginning of experiment.
Fig. 4. Taken after 150 minutes from beginning of experiment.
Fig. 5. Taken after 18 hours from beginning of experiment.

(The times stated are approximate.)

irregular convoluted formation, well illustrated by the photographs. The zone of precipitation in the positive wave is situated, in the early part of the experiment, near the positive electrode, but recedes from this electrode as the experiment proceeds. The approaching negative particles attracted by the anode are discharged, precipitated, reversed in sign and redispersed by the advancing acid wave. The positively charged particles behind the wave are strongly repelled by the positive electrode and tend to sweep before them the uncharged particles in the isoelectric state in the anode wave. Behind the dense anode wave proper we see, in the photograph, evidence of a smooth zone of dispersed particles, behind which is a clear acid zone. If the experiment be performed sufficiently slowly, the convolutions will not appear in the anode wave. On strongly increasing the potential across the cell, however, the positive wave immediately develops an undulating character. We believe that the precipitation of the particles is necessary before these undulations can occur. The change of sign of the particles apparently does not take place absolutely simultaneously, so that some of them are more repelled from the positive electrode than others. In this way, possibly, the convolutions may arise.

(3) In front of the positive wave there is a clear zone which is not present in advance of the negative wave.

(4) Ultimately the two waves meet and precipitate practically together near the centre of the cell. In this position a neutral zone is formed by the neutralisation of the acid and alkali of the respective waves. In this zone, naturally, the precipitation of the protein particles occurs. This is well shown in the photographs, where the positive and negative waves are united in a zone of common precipitation.

(5) If the current be stopped while the waves are advancing, they show a marked tendency to diffuse back again in the opposite directions to the directions in which they are travelling, showing that the charges of the electrodes are necessary for the movements of the waves. If the current be stopped after the common precipitation has occurred near the centre of the cell, then the precipitated waves remain unaltered for a considerable time.

(6) Slight evolution of gas, due to electrolysis, is seen to occur at each electrode.

A further experiment was carried out, in which strips of neutral litmus paper were placed in a cell of the macroscopic type. The paper should not contain an excess of alkali. This experiment showed that the acid and alkali waves preceded the visible positive and negative waves respectively for a small distance and showed the close relationship between these waves and the changes of reaction. It likewise showed that the zone of common precipitation occurred where the acid and alkali waves met. As the colloid waves recede from their respective electrodes, their velocities diminish. If the colloid be made slightly more alkaline by the addition of free alkali, the positive wave

has an increased lag in emerging and the zone of common precipitation is shifted towards the anode. On the other hand, if the colloid be slightly acidified, the positive wave emerges first and the appearances are reversed, the zone of common precipitation being now moved towards the cathode.

SUMMARY.

Photographs of a modified Hardy experiment are described, which demonstrate simply some of the electrical properties of amphoteric colloids. The experiment should be helpful for class demonstration purposes.

REFERENCE.

Hardy (1899). *J. Physiol.* **24**, 288.

CCXVII. A PRELIMINARY NOTE ON A PROLAMIN ISOLATED FROM A LEGUME SEED.

By ALI HASSAN and MOHAMMED KAMIL ABU BASHA.

From the Biochemical Laboratory, Department of Physiology, Faculty of Medicine, Cairo.

(*Received September 21st, 1932.*)

The majority of the inhabitants of Egypt live on a variety of maize bread in the making of which a small proportion of the highly mucilaginous and bitter seeds of the legume known as fenugreek is added. Usually 2–5 % of fenugreek is used, but this small quantity has the advantage not only of conferring on the moist mixture better binding properties thus facilitating the production of the characteristic wafer-like loaf, but definitely makes the finished loaf more appetising.

While investigating the nutritional value of the protein mixture of this maize-fenugreek bread compared with that of ordinary wheaten bread, an alcohol-soluble protein was isolated from the dried powdered fenugreek seeds. This discovery appeared to be interesting since, according to Osborne [1924], alcohol-soluble proteins have so far been found only in the cereals.

Preparation.

After eliminating any cereal grains or other foreign materials, if present, the fenugreek seeds are ground in a clean strong laboratory mill, and the flour is subsequently extracted completely with ether. The ether extraction can be omitted, its only advantage being that it minimises loss in the later stages of the process as it gives a purer product.

The air-dried ether-extracted flour is now boiled with 70–72 % alcohol (1500 cc. to every 500 g.) on a water-bath. After 1 hour the hot contents of the flask are filtered rapidly through a Büchner funnel. The residue is extracted once more with the same strength of alcohol for another half hour, after which it is filtered hot as before.

On cooling, a yellowish-white precipitate begins to form and is allowed to settle in a covered vessel for 16 to 24 hours; the supernatant liquid is then carefully syphoned off and the moist precipitate centrifuged and subsequently washed with cold 70 % alcohol followed by 96 % until the washings are no longer yellow. The residue is then taken up in liquid phenol and again centrifuged. The greater part dissolves in the phenol giving a clear reddish-brown solution. The residue is treated two or three times more with phenol before it is discarded.

The phenol solutions are treated with 96 % alcohol until precipitation is maximum, and the precipitate is repeatedly washed with the same strength of alcohol until the last traces of phenol are removed. Many washings are required to effect this. Finally the precipitate is washed with absolute alcohol on a Büchner funnel and then with ether. The ether is allowed to evaporate and the dry material is powdered and kept in a desiccator over calcium chloride.

Properties.

The material thus obtained is an amorphous, tasteless and odourless white powder. It is very slightly soluble in distilled water, more soluble in dilute HCl but it dissolves freely in dilute alkali. Its solubility in alcohol is somewhat peculiar. It only dissolves in hot 70 % alcohol and separates on cooling; it is insoluble in 96 % alcohol. A solution of the material in dilute alkali gives the following reactions:

Biuret	Violet
Xanthoproteic	Positive
Millon's	,,
Rosenheim's tryptophan test	Very faint, on standing
Sulphur test	Positive
Molisch's	Negative

It is precipitated by picric acid and also by hydroferrocyanic acid and by half saturation with ammonium sulphate. Half saturation with magnesium sulphate produces a definite turbidity, but complete precipitation is only brought about by full saturation with this salt. The isoelectric point lies approximately at p_H 5·3.

The material contains 17·18 % N and 1·115 % S.

The yield is very small. Roughly, 0·2–0·5 g. of material is obtained from 100 g. of flour. The major part comes out in the first extraction, and there is, therefore, no necessity to extract more than twice with 70 % alcohol.

Conclusions.

The above description shows that the material isolated from fenugreek by treating its dry powdered seeds with boiling 70 % alcohol is a protein. This protein, for which the name "helbin" is proposed (from "Helba," Arabic for fenugreek), might be classed with the gliadins for, like the gliadins it is soluble in boiling 70 % alcohol and in phenol and is precipitated from its phenolic solution by the addition of ethyl alcohol, acetone, amyl alcohol or ether. It differs, however, from the gliadins of wheat and maize, for example, in being insoluble in cold 70 % alcohol. It is therefore of interest to determine the exact relations of helbin to the known gliadins. The anaphylactic reaction should be of great help in this connection and it is intended to study the serological reactions of this protein and to compare them with those of the other gliadins.

SUMMARY.

1. A compound soluble in hot 70 % alcohol and possessing the characters of a protein has been isolated from the seeds of the legume known as fenugreek.

2. It differs from the known gliadins in being insoluble in cold, but soluble in hot 70 % alcohol.

3. Like the gliadins, this protein is soluble in phenol and is precipitated from its phenolic solution by the addition of ethyl alcohol, ether, *etc.*

We wish to thank Prof. G. V. Anrep for his encouragement and interest in the work.

REFERENCE.

Osborne (1924). The vegetable proteins. (Longmans, London.)

CCXVIII. THE ANTISCORBUTIC VALUE OF FRESH LIME JUICE.

By ALI HASSAN and RIZK BASILI.

From the Biochemical Laboratory, Department of Physiology, Faculty of Medicine, Cairo.

(*Received September 21st, 1932.*)

Studying the antiscorbutic value of the lime, Chick, Hume and Skelton [1918] found that the majority of the preserved lime juices examined were valueless as a source of this important dietary factor. This valuable information satisfactorily explained the then growing lack of confidence in preserved lime juice as a protective or therapeutic agent against scurvy.

While determining the antiscorbutic content of preserved lime juice sent by the Department of Commerce and Industry, complete confirmation of the above observation was obtained. For none of the preserved juices subjected to the test contained any detectable quantity of the antiscorbutic principle.

With fresh lime juice, however, our results differed markedly from those reported by the same investigators. According to them, the minimum protective dose of fresh lime juice for a guinea-pig is 5 cc. daily, while in our case complete protection was attained with a much smaller dose. As a matter of fact our findings show clearly that there is no difference between the antiscorbutic content of the truly fresh ripe lime and that of the fresh lemon.

From the point of view of lime-growing countries like Egypt, this result is quite important both nutritionally and commercially. In this country fresh limes and lime juice are much preferred to lemons which are grown here on a limited scale. It follows that one may have to fall back on the lime in case of emergency, and it is, therefore, of great importance to know the exact place which must be assigned to this fruit among the antiscorbutic agents.

Before discussing the possible causes of the difference between our result and that observed by Chick and collaborators, a description of the experiments which led to this result will be given below.

Technique.

The diets. As a scurvy-producing diet the following ration was prepared: caseinogen 8, calcium carbonate 1·5, sodium chloride 1, oats 55, and bran to 100. This diet was given *ad lib.* supplemented with 0·5 cc. cod-liver oil daily per animal. When an adequate supply of the antiscorbutic was provided, the guinea-pigs grew at a good rate and appeared quite healthy. When, on the other hand, no antiscorbutic was given the animals exhibited, both during life

and at autopsy, a typical picture of scurvy. Death occurred 24 to 32 days from the commencement of the feeding and was always preceded by a decline in weight starting near the end of the second week.

For certain reasons a modification in the diet was introduced during the course of the experiment. The oats were replaced by an equivalent quantity of barley after proving that the change in no way influenced the scurvy-producing properties of the ration.

Experimental animals. Young guinea-pigs as obtained from a dealer were employed for the test. They were usually kept on greens and bread for 2 days before starting the feeding. Animals weighing 300–400 g. were, as a rule, selected, but in a very few cases guinea-pigs of weights slightly outside this range had to be used. The pigs were housed in capacious metal cages with wood shavings as bedding. Food and water were served *ad lib.* and the cod-liver oil and the juice under examination were fed by hand from a pipette.

It was found necessary to keep the mouth of the animals and the surrounding area of the face perfectly clean. When this was neglected stomatitis usually developed especially in those receiving a highly sugared juice. The food consumption of animals suffering from this disorder generally dropped, possibly from lack of appetite or from pain due to the inflamed condition of the mouth.

Preparation of the juice. The juice was prepared fresh daily from ripe fruits recently picked. Actually none of the fruits employed was older than 2 days at the outside. After being cut into halves, the limes were squeezed by hand into a muslin-covered funnel, and, to the strained juice, $CaCO_3$ and sugar were added in the proportion of 15 cc. juice, 0·5 g. $CaCO_3$ and 2 g. sugar and when all the sugar had dissolved, the volume was made up to 20 cc. with water. The mixture was then centrifuged and the supernatant fluid employed for the experiments.

The diagnosis of scurvy. A word may be said here about the plan adopted for the diagnosis of scurvy. It is intended to discuss only the *post mortem* findings. In severe cases, no difficulty is experienced in diagnosing the condition, for typical subcutaneous and intramuscular haemorrhages can be easily detected macroscopically in the characteristic sites. Similarly joint swellings, beading of the rib junctions, fragility of the long bones and looseness of the front teeth, which are constant accompaniments of the vascular changes, are also easily detectable. There are cases, however, which present a less complete picture of the disease. In these, haemorrhages are usually absent. Swellings of the joints and beading of the costochondral junctions, with or without fragile bones and loose teeth, are the only pathological changes to be seen. The diagnosis of these cases was not so easy, especially as similar bone changes have been ascribed to other deficiencies [Delf and Tozer, 1918]. Nevertheless these cases have been classed as mild scurvy, for they occurred only in animals receiving either an inadequate dose of the antiscorbutic or none at all. It will be remembered that each animal received by hand a daily dose of 0·5 cc. of tested cod-liver oil and that those getting an adequate supply

of the antiscorbutic principle were entirely free from any bone changes whatsoever. Another point, which seems to support the view that these bone changes are scorbutic in origin, is that in the same batch of guinea-pigs on the same insufficient dose of the antiscorbutic one encounters both types, that is, animals exhibiting at autopsy nothing but bone lesions and others showing haemorrhages as well.

The absence of scurvy was determined by the behaviour of the animals during life, especially as regards growth and general health, and by a careful study of the different systems after death.

A summary of the results obtained with fresh lime juice is embodied in Table I.

Table I. *Source of antiscorbutic principle. Fresh lime juice.*

Daily dose in cc.	Initial weight g.	Duration of exp. days	Result
0	250	25	Dead, scurvy
0	250	30	,,
0	300	32	,,
0	400	24	,,
0·5	350	46	,,
0·5	354	47	,,
0·5	370	49	,,
0·5	318	57	,,
0·75	440	66	Dead, no haemorrhages, fragile bones, enlarged knee joints and rib junctions
0·75	338	104	Killed, chronic scurvy, knee joints ankylosed
1·0	333	51	Dead, moderate scurvy
1·0	300	62	,,
1·0	340	93	Killed, mild scurvy, slight bone changes only
1·5	353	31	Dead, bronchopneumonia, no scurvy
1·5	358	93	Killed, no scurvy
1·5	343	44	Dead, liver abscess, no scurvy
1·5	320	93	Killed, no scurvy
1·5	350	93	,,
2	270	93	,,
2	300	93	,,
3·5	380	93	,,
3·5	250	93	,,

From Table I above, it can be clearly seen that the minimum protective dose of fresh lime juice, obtained as described earlier, is 1·5 cc., and that partial protection is conferred even by 0·5 cc. which was the smallest dose tried.

Having established this point, it became interesting to define the relation of the Egyptian lime to that of the West Indies, since the latter fruit was employed in most of the previous work on this problem. There is no doubt that Egyptian limes belong to the group of the *Citrus medica* var. *acida* and, as a matter of fact, are, to the layman, indistinguishable from the West Indian limes, few trees of which are grown in the gardens of the Horticultural Section of the Ministry of Agriculture.

Many explanations can be suggested to account for the difference between the antiscorbutic value of fresh lime juice as reported here and that found by

other workers. But it appears that the difference is most probably due to the fact that, in previous work, the lime juice employed was obtained from relatively much older fruits and was sometimes kept for as long as 2 months before examination. If this explanation is true, the juice of West Indian limes examined under the conditions detailed above, ought to have an antiscorbutic value similar to that of fresh Egyptian limes. The results reported by Davey [1921] for fresh lime juice make this conclusion highly probable. With a special sample of lime juice, the minimum protective dose appeared to be less than that reported earlier by Chick *et al.* [1918]. This result also supports the view that, in lime juice, the antiscorbutic principle undergoes deterioration at a much quicker rate than in the case of other citrus fruits such as the lemon.

Owing to the close of the season, it was not possible to obtain direct information on this point. It was, however, decided to make some use of the few West Indian limes left on the trees, for comparison with Egyptian limes on the lines of the work done by previous investigators. West Indian limes grown locally and Egyptian limes were subjected to examination at one and the same time. In the preparation of the juice, quite fresh and ripe fruits were used. These were cut into two and squeezed by hand; and to the juice, strained through muslin, sodium benzoate was added as a preservative. The juice was kept in a refrigerator throughout the experiment and fed as such to the animals from a burette. The results obtained are given in Table II.

Table II.

Source of antiscorbutic	Initial weight g.	Duration of exp. days	Result
Egyptian Limes			
1 cc. daily	303	41	Died, bone changes only
,,	358	42	Died, moderate scurvy
2 cc. daily	304	41	Died, bone changes only
,,	315	40	,,
3 cc. daily	350	45	,,
,,	270	51	Died, scurvy
West Indian Limes			
1 cc. daily	344	30	Died, moderate scurvy
,,	304	36	,,
2 cc. daily	360	35	,,
,,	364	56	Died, scurvy, severe
3 cc. daily	450	42	Died, moderate scurvy
,,	432	40	,,

Table II shows that the antiscorbutic principle undergoes deterioration in the juice obtained from Egyptian limes and kept in the refrigerator during the experiment, for 3 cc. failed to protect the animals against scurvy (*cf.* Table I). Compared with the West Indian lime juice, the animals on the Egyptian lime juice lived slightly longer. It is not possible, however, to attach any importance to this difference at present.

Discussion and Conclusions.

In determining the antiscorbutic value of fresh lime juice, Chick *et al.* [1918] employed imported limes which took 4 to 8 weeks in transit and the squeezed juice was fed for 2 months, that is, until a fresh consignment was received. Davey [1921], following a similar technique, arrived at practically the same conclusions regarding fresh lime juice.

In the first set of experiments presented above, the juice was prepared fresh daily from ripe fruits not more than 2 days gathered. If we agree that there is no essential difference between the lime grown in Egypt and that of other localities, the low antiscorbutic value reported previously for fresh lime juice can be ascribed to deterioration of the antiscorbutic principle. There seems to be no doubt that loss of this factor takes place at a much greater rate from lime juice than from lemon juice, and, as a result of the present work, one may conclude that loss starts in the intact fruit. Davey [1921] obtained slightly better results with green than with ripe limes. It thus appears that loss of the antiscorbutic principle commences with the process of ripening.

The experiments with the West Indian and Egyptian limes only show that in both juices the antiscorbutic principle is not stable for a long time. During the experimental period (approx. 2 months) deterioration was such that 3 cc. failed to protect against the disease.

Summary.

1. The antiscorbutic value of lime juice, prepared fresh daily from ripe Egyptian limes not more than 2 days gathered, was found to be 1·5 cc. per guinea-pig daily.

2. 3 cc. of "fresh" lime juice, prepared under the same conditions but kept in the refrigerator during the whole course of the experiment (approx. 2 months), failed to protect the guinea-pig against scurvy.

We wish to thank the Director of the Horticultural Section and Abdel Badei Eff. of the Gebel Asfar Farm for supplying us with most of the material. Our thanks are also due to Prof. G. V. Anrep for the interest he took in the work.

REFERENCES.

Chick, Hume and Skelton (1918). *Lancet*, ii, 735.
Davey (1921). *Biochem. J.* **15**, 83.
Delf and Tozer (1918). *Biochem. J.* **12**, 416.

CCXIX. THE EFFECT OF HYDROLYSIS ON THE SUGARS OF NORMAL URINE.

By ALI HASSAN and MOHAMMED ABDEL SALAM EL AYYADI.

From the Biochemical Laboratory, Department of Physiology, Faculty of Medicine, Cairo.

(*Received September 21st, 1932.*)

According to various investigators, the reducing power of normal urine may, after acid hydrolysis, follow one of three courses. It may either diminish, increase or remain unaltered. If we accept the view that normal urines usually contain variable quantities of ill-defined polysaccharides, the augmentation of the reducing power after hydrolysis can be satisfactorily explained. The frequency and magnitude of the rise suggest that, under normal conditions, the excretion of such polysaccharides cannot be very large, although the polysaccharide content of the urine can be appreciably increased by the ingestion of certain carbohydrates such as dextrin. Folin and Berglund [1922] definitely demonstrated the presence of erythrodextrin in the urine of a healthy individual after giving 200 g. of this material by mouth. In this particular case the increase in the reducing power after hydrolysis was very marked.

The explanation of the diminution of the reducing power after hydrolysis is, on the other hand, not so easy. Little is known about the nature of the non-carbohydrate reducing substances of urine apart from uric acid, urochrome and creatinine. There is reason to believe however that other reducing substances also occur. In the methods employed for the determination of the sugars of normal urine attempts are made, it is true, to remove these non-carbohydrate reducing substances but the inefficiency of these procedures is apparent when the methods are compared; no two methods agree when applied to one and the same urine. It follows that the diminution of the reducing power of normal urine, which sometimes occurs after hydrolysis, may be due to partial destruction of the sugars or of the non-carbohydrate reducing materials or of both. It is obvious that a diminution of the reducing power can be observed only in urines almost or completely free from polysaccharides. That such diminution is due to partial destruction of the sugars has often been suggested, but no direct experimental evidence, as far as we are aware, has been brought forward in support of this view.

Normal urine probably contains at least two sugars one of which is glucose whilst the other, which occurs in relatively greater proportion, has not yet been identified. This latter sugar will, for convenience, be called *iso*maltose, which name was given to it some time ago because of the occasional

similarity of its osazone to Fischer's *iso*maltosazone. Then, if we accept the view that the diminution of the reducing power is due to sugar destruction, it would be of interest to investigate the extent of the destruction as far as each sugar is concerned. This result should be more satisfactorily attained if some procedure were adopted by which the complete removal from the urine of the dextrin-like bodies and the other complex polysaccharides can be assured. The presence of these bodies must naturally disturb the result by masking the destructive action of the acid on the pre-existing sugars. Such mutually antagonistic effects must obtain in urines in which the total reduction is not altered by hydrolysis.

While it is possible to devise a means for the complete removal of the complex carbohydrates, the removal of sucrose is not so easy. Fortunately, however, sucrose is not frequently met with in the urine, besides which it is possible to limit the examination to urines of subjects known not to have partaken of foodstuffs containing this disaccharide.

Working on these lines and with the help of the osazone reaction it has been possible to demonstrate that hydrolysis by acid destroys a large part of the sugar called *iso*maltose while glucose seems to be unaffected.

Experimental.

All the urines investigated were collected from young healthy individuals and in every case the food taken by the subject was noted. About 150 samples were examined and these represented odd specimens as well as 24 hour collections.

Every sample was first treated with charcoal, this treatment being an essential step in the preparation of the osazones of the urine-sugars. However, it must be remembered that charcoal not only removes the substances which normally interfere with the proper crystallisation of the osazones, but unfortunately also removes a variable quantity of the urine-sugars. After having compared the action of five specimens of charcoal, it was found that the B.D.H. decolorising charcoal was most suitable, since it interfered least with the urine-sugars and gave a clear colourless filtrate.

The charcoal treatment consisted in adding 8 g. charcoal to 100 cc. urine and filtering after allowing the mixture to stand for 5 minutes. The clear colourless filtrate was employed for the determination of the reducing power and the preparation of the osazone both before and after hydrolysis.

The hydrolysis of the charcoal filtrate was done with the help of HCl in a manner similar to that recommended by Folin and Berglund [1922], 20 cc. were measured in a tube graduated for the purpose, 2 cc. 10 % HCl were then added and the contents heated in a boiling water-bath for 75 minutes. After thorough cooling, the hydrolysed filtrate was neutralised with a few drops of a strong solution of NaOH. At first phenolphthalein was used as an indicator but later its use was discontinued and the alkali was very carefully added till

the phosphate precipitate just began to form. Then water was added to bring the volume back to the 20 cc. mark.

The reducing power of the filtrate was determined by Shaffer and Hartman's method [1920] and in the preparation of the osazone the method described by Hassan [1928] was followed.

Changes in the reducing power.

As has been stated previously about 150 urines were examined and with the exception of three, all showed a marked diminution in the reducing power after hydrolysis. Table I gives the figures obtained in a few cases.

Table I. *Effect of hydrolysis on reducing power.*

Total reduction in mg. glucose per litre	
Before hydrolysis	After hydrolysis
412	350
322	150
266	124
120	34
370	350
158	132

The samples which showed an increase after the acid treatment constituted 2 % of the total and in all sucrose was proved to be present. As a matter of fact the subjects from whom these three specimens were obtained admitted partaking of meals containing pastries sweetened with cane sugar syrup.

The causes of this diminution may be numerous, but the present work has been limited to investigating the influence of the acid on the urine-sugars. It was thought that the osazone reaction would be helpful in this respect. This reaction is not only capable of demonstrating changes in the quantity of the sugars but it can also show, within reasonable limits, variations in their proportions. The osazones of the sugars of the charcoal-treated filtrate have therefore been prepared before and after hydrolysis and the products compared.

In order to have a clear idea of the following comparison, the reader is referred to the paper by Hassan [1928] in which a description of the osazones of the sugars of normal urine is given. There it is shown that these compounds are crystal mixtures formed of at least two components, and that, under suitable conditions, these crystal mixtures can be split into two osazones having distinct characters. Osazone I crystallises out in rosettes formed of broad flat needles, is soluble in hot water and melts at about 152°, and osazone II is identical with glucosazone.

On comparing the microscopic appearance of the two products, a striking difference was immediately noticed. The osazones before hydrolysis presented the usual crystal mixtures in which the proportions of the two osazones varied greatly. Those obtained after hydrolysis showed, on the other hand, a marked diminution in the broad needles and a tendency to aggregate in sheaf formations instead of the usual rosettes and in a large number of the samples the crystal mixtures were replaced by glucosazone sheaves (see Plate VIII).

This difference can only be due to one of two factors. Either hydrolysis increases the glucose content of the filtrate or destroys the urine-sugar which gives rise to osazone I. In the former case the glucosazone character will predominate by virtue of the increased concentration of glucose, while in the latter the destruction of the *iso*maltose will leave the field free to the pre-existing glucose. It can, however, be argued that the change in the crystal shapes might simply be due to changed conditions of crystallisation and not to an alteration in the proportions of the two sugars. This objection is quite reasonable because it is very unusual, when the osazones of the sugars of any one urine are recrystallised, to get the same crystal shapes. The crystal mixtures obtained on recrystallisation can assume a large variety of appearances depending on the rate of crystallisation and so forth, but it is always possible to see that in the new crystal mixtures the proportions of the two components remain practically the same. The difference cannot be due to the formation of new sugar because the charcoal employed adsorbs the dextrin-like bodies, at least in the concentration in which they occur normally. Moreover the result reported above, concerning the uniform diminution of the reducing power after hydrolysis, is in itself a proof that the charcoal-treated filtrates are free from compounds capable of giving rise to fresh reducing substances on hydrolysis; sucrose is, of course excluded.

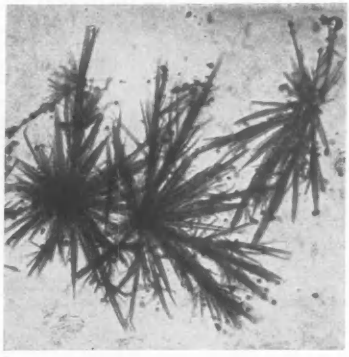
The experiment described above shows that the diminution in the reducing power and incidentally the change in the appearance of the crystal mixtures are probably due to destruction of *iso*maltose, *i.e.* the sugar which gives rise to osazone I. If this conclusion is correct then we should observe the following changes in the osazones of the hydrolysed filtrates.

(*a*) The osazone yield should be less.

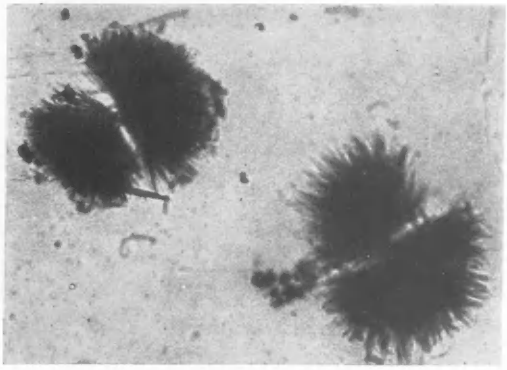
(*b*) There should be a rise in the M.P. of the crystal mixtures.

(*c*) The solubility of the crystal mixture should be less.

If destruction of the sugars is taking place during hydrolysis, one naturally expects to get less osazone formed after the acid treatment. To show that this is actually the case is by no means easy because of the difficulty of purifying the small yield without incurring great loss. After many trials we adopted the following procedure which, though it cannot give a pure product, is quite adequate for comparative purposes. The osazones were prepared under exactly similar conditions from the filtrates before and after hydrolysis. The precipitate was then filtered in a weighed Gooch crucible, washed with cold water and treated with as little chloroform as was necessary to wash away the oxidation products of the phenylhydrazine. The same volume of chloroform was used in both cases and the washing was stopped when the chloroform was free from colour. The crucible and contents were then dried to a constant weight. In all cases the osazone yield weighed less after hydrolysis (see Table II). The difference, however, did not always run parallel with that found for the reducing power. A possible explanation of this divergence will be discussed later on.



(*a*)



(*b*)

Osazones obtained from the same urine filtrate.
(*a*) Before, (*b*) after hydrolysis.

Table II. *Osazone yield.*

Weight of osazone in mg.	
Before hydrolysis	After hydrolysis
2·4	2·0
3·8	1·0
4·8	2·0
5·0	2·6
4·6	3·0

The melting-point and solubility.

Crystal mixtures vary in their M.P. and the variation depends on the proportion of each sugar present. Those containing more glucosazone would be expected to have a higher M.P. than those with relatively more of osazone I. If hydrolysis destroys the sugar giving rise to osazone I, that is, the osazone with the lower melting-point, a rise in the M.P. of the resulting osazone should follow. To prove this, an adequate quantity of the charcoal filtrates (50 to 100 cc.) was used for the preparation of the osazones and the melting-point of the crystals, purified by recrystallisation was determined. The following figures represent some of the results obtained.

Melting-point determinations	
Before hydrolysis	After hydrolysis
162°	181°
163°	186°
161°	180°

As would be expected the solubilities of the products exhibited unmistakable differences. In any one urine, the osazones obtained before hydrolysis were more soluble in hot water and in cold alcohol than those obtained after hydrolysis.

Discussion and conclusions.

Whether *iso*maltose or some other compound gives rise to osazone I, the parent substance seems to be slowly destroyed by acid hydrolysis, whilst glucose is unaffected under the same conditions. This assumption is supported by the microscopic appearance of the crystal mixtures in which an increase rather than a decrease of the glucosazone element is noted after hydrolysis. This increase is, however, only apparent, for the diminution in osazone I will not only give the glucosazone a chance to impress more of its own characters on the crystal mixtures but may, if the diminution is great, even allow it to form typical glucosazone. Moreover, subjecting an aqueous solution of glucose containing 40 mg. per 100 cc. to acid hydrolysis did not affect its reducing power. The destruction of *iso*maltose does not account, in many cases, for the total diminution in the reducing power and, as a matter of fact, there is no reason to believe that it can. Even in the charcoal-treated filtrate, the sugars are not the only reducing substances present. The lack of parallelism, referred to above, between the diminution in the reducing power and the

weight of the osazone found after hydrolysis points to the possible existence in the filtrate of non-carbohydrate reducing substances, and it is highly probable that some of these substances undergo destruction by acid as well.

SUMMARY.

1. The reducing powers of the charcoal-treated urines of about 150 healthy subjects were determined before and after hydrolysis with 10 % hydrochloric acid.

2. With the exception of three cases, in which sucrose occurred, a diminution in the reducing power was observed.

3. Evidence is given to show that this diminution is due, at least in part, to destruction of the so-called *iso*maltose occurring normally in urine.

We wish to thank Prof. G. V. Anrep for his encouragement and his interest in the work.

REFERENCES.

Folin and Berglund (1922). *J. Biol. Chem.* **51**, 210.
Hassan (1928). *Biochem. J.* **22**, 1332.
Shaffer and Hartmann (1920). *J. Biol. Chem.* **45**, 365.

CCXX. A SIMPLE METHOD FOR THE DETERMINATION OF THE MELTING POINT OF FATS AND ALLIED SUBSTANCES.

By MOHAMMED ABDOU ABBASSI.

From the Biochemical Laboratory, Physiology Department, Faculty of Medicine, Cairo.

(*Received September 27th, 1932.*)

In the method to be described, use is made of the fact that fats and similar substances are, as a rule, bad conductors of electricity. A thin film of the substance whose M.P. is to be determined is interposed in the path of an electric current. Heat is then gently applied, and when the substance melts, the circuit is immediately completed and a bell is rung.

As seen from Fig. 1, the apparatus required is quite simple. The only part that needs description is the film-terminal (*A*). This is made by sealing the end of a piece of glass tubing, 0·5 cm. in diameter and about 20 cm. long, round a short length of platinum wire, the outer end of which should be filed flush with the surface of the sealed end. If allowed to protrude much beyond the outer surface, a large quantity of material would be required to coat the wire efficiently, with the result that the insulating film would be unduly thick. The flat cork (*C*) clamped to a retort stand, carries two holes to accommodate the film-terminal and the thermometer. The other parts of the apparatus comprise a Leclanché cell (*D*) or some other suitable source of current, an electric bell, a tall pyrex beaker three-quarters full of clean mercury, a tripod and a micro-burner.

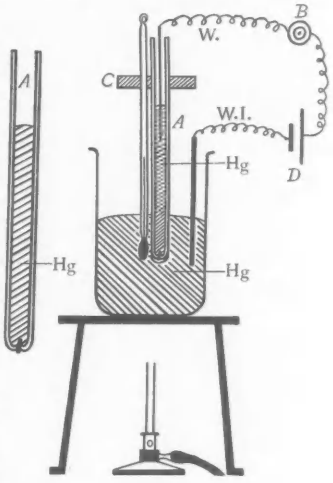


Fig. 1.

To carry out a determination, the substance whose melting-point is to be determined, is just melted in a clean dry test-tube and the clean dry bottom end of the film-terminal is dipped in the molten mass and quickly removed. The resulting film which need not cover more than the exposed end of the platinum wire and a small area of the glass surface round it, is then allowed

to solidify completely. The film-terminal is fixed in the cork in such a way that the end carrying the film stands close to, and level with the bulb of the thermometer. Both are then dipped in the cold mercury in the beaker to about 4 cm. from the surface and about this distance from the bottom. Clean mercury is now poured inside the film-terminal, and the wires from the cell (*W.* and *W.I.*) are brought into contact with the mercury in the film-terminal and that in the beaker as shown in the diagram. The beaker can now be heated slowly and the temperature at which the bell commences ringing is noted. This temperature represents the M.P. of the material for it is assumed that immediately melting takes place, the upthrust of the mercury pushes the molten mass up to the surface and contact is at once made between the mercury in the beaker and that in the film-terminal through the exposed platinum wire.

To ascertain the exact M.P., the experiment should be repeated at least twice, taking the usual precaution of heating slowly before the expected M.P. is reached. After each determination the end of the terminal is wiped with a soft cloth moistened with a proper solvent and then dried in alcohol followed by ether. It is advisable to have many such terminals ready for use in a clean covered jar.

The melting-points of a large number of suitable substances were repeatedly determined with encouraging results and the method was then compared with some of the methods already in use. The following table gives the melting-points of six samples of paraffin obtained by the present method (I) and by the help of Mason's melting-point apparatus (II).

Substance	I. M.P.	II. M.P.
A	55·4°	55·1°
B	59·3	59·3
C	47·9	47·8
D	43·9	43·9
E	48·7	47·8
F	51·5	51·1

Each result is the mean of at least three determinations and it will be seen that the figures in the two columns agree fairly closely.

This method is decidedly less laborious and certainly much cleaner than most of the methods in general use. The use of a stirrer is unnecessary because mercury is a very good conductor of heat, and tests have shown that, with slow heating, the temperatures taken at different places in the mercury-bath, are fairly even. The application of the method is naturally limited to substances capable of forming a thin compact non-conducting film, such as the fats, waxes, paraffin *etc.*

It is a great pleasure to me to thank Dr Ali Hassan for his help and encouragement.

CCXXI. HYDROGENLYASES.

II. SOME FACTORS CONCERNED IN THE PRODUCTION OF THE ENZYMES.

By JOHN YUDKIN.

From the Biochemical Laboratory, Cambridge.

(*Received September 24th, 1932.*)

It has long been known that yeasts can be trained to ferment sugars upon which they are unable to act previous to training. The work of Dubourg [1899] on the training of yeasts to act upon cane sugar was criticised by Klöcker [1900]. But Dienert [1900] gave an account of some work on the acclimatisation of yeasts to galactose which is noteworthy. After growing yeasts on galactose, which, at the outset, they were not able to ferment, it was found that in circumstances where growth was impossible galactose was now fermented. The training was very rapid, 24 hours being sufficient to produce this property. The acclimatisation was lost only after growing on some other fermentable sugar for a long time. Yeasts which previously were unable to grow at all with galactose as the only carbon source, were acclimatised to galactose by growing in the presence of glucose and galactose. They were then able to grow with galactose as the sole carbon source.

Harden and Norris [1910] and Willstätter and Sobotka [1922] also trained yeasts to ferment galactose. The former showed that the press juice from acclimatised yeasts was able to ferment galactose. Abderhalden [1925] found that this property is not lost even if the yeast is grown for a long time on other carbon sources.

Similar work has been done on many other reactions induced by yeasts, moulds and bacteria. Thus Klotz [1906] trained a bacterium of the colon group to ferment lactose, and Twort [1907] was able to train *Bact. typhosus* to ferment lactose, and *Bact. dysenteriae* (Flexner) to ferment sucrose.

Karström [1930] described the adaptation of several species of bacteria to certain substrates. He concluded that bacterial enzymes may be classified as either constitutive or adaptive. The former are invariably present in the bacteria; the presence of the latter depends on the presence, in the medium on which the culture is grown, of the substrate upon which they act. Sometimes, the presence during growth of a certain substance inhibits the formation of an enzyme which is present in cultures grown on other media. For example, one of Karström's strains of *Bact. coli* contained saccharase when grown

on any sugar other than glucose, or when grown in the absence of sugar. Growth on glucose, however, gave a culture which had no saccharase.

It is necessary here to distinguish between "training" and "adaptation." First, it is clear that a given strain of bacterium can perform certain chemical reactions and cannot perform certain others, and that these properties normally remain constant. For example, a strain of *Bact. coli* growing on peptone and glucose ferments the glucose, but growing on peptone and sucrose does not ferment the sucrose. The phenomenon which has been called "training" consists in obtaining a strain which carries out a reaction which it was previously incapable of doing, by growing it for a large number of sub-cultures in a medium containing the new substrate. The new reaction then becomes a normal property of the strain. The phenomenon called "adaptation" is totally different, and is concerned with reactions which are already normal to the bacteria concerned. Of these reactions, some are catalysed by enzymes which are invariably present in the cells, on whatever medium they are grown; some by enzymes which are formed only when the cells are growing in the presence of the particular substrate. These two types are called respectively "constitutive" and "adaptive" enzymes. For example, one of Karström's strains of *Bact. coli* was able to ferment maltose immediately it was inoculated into a medium containing this sugar. It did not require to be trained to perform this reaction. However, a washed suspension of the bacteria grown in the absence of maltose was not able to ferment it, whilst a suspension of bacteria which had been grown in its presence did induce a fermentation, that is to say the enzyme is "adaptive." The training of organisms may proceed very rapidly as when Dienert [1900] acclimatised yeast to galactose in 24 hours. But once the organism has been trained, the newly acquired property is lost very slowly, if at all [Abderhalden, 1925]. On the other hand, an adaptive enzyme, which seems to be formed as a response to a chemical stimulus, is no longer produced when the stimulus is removed. This point is more fully discussed later.

Following the work of Karström, Stephenson and Stickland [1932] investigated the hydrogenlyases of bacteria, *i.e.* enzymes which liberate molecular hydrogen from formate, glucose, *etc.* It was found that washed suspensions of *Bact. coli* and *Bact. lactis aerogenes* possess formic hydrogenlyase when sodium formate is present in the medium on which they are grown, but not in its absence. The following work was undertaken with a view to determining further the factors concerned in the formation of these adaptive enzymes.

Experimental.

In all the experiments to be described, the bacteria in question were inoculated from an 8-hour culture in caseinogen digest broth into the medium concerned. The amount of sodium formate, glycerol or glucose added was always 0·5 %. Growth was allowed to continue for 15–18 hours (unless

otherwise stated) at 37°. The culture was washed twice with Ringer's solution by centrifuging and then made up in uniform suspension in Ringer's solution. The resulting preparation of "non-proliferating bacteria" was investigated as follows in the Barcroft manometer at 40° [Stephenson and Stickland, 1932]. 1 cc. of bacterial suspension and 1 cc. of $M/20$ phosphate buffer (p_H 7·0) were placed in each cup. In one cup was placed 1 cc. of the substrate, usually in $M/10$ solution, and in the other 1 cc. of water. The inner small cups were half filled with 40 % NaOH for absorption of carbon dioxide. The apparatus was twice evacuated and each time filled with nitrogen which had been passed over heated copper to remove small amounts of oxygen.

After equilibration readings were taken at intervals of 5 or 10 minutes.

In the case where gas is being evolved from glucose or glycerol, the rate is constant for a long time—certainly more than an hour. However, when formic hydrogenlyase is under investigation, the rate is rarely constant for longer than 20 minutes. The reason for this falling off is partly due to the alkalinity which soon manifests itself, and partly due to the fact that the formic hydrogenlyase has a low affinity [Stephenson and Stickland, 1932]. Thus the concentration of formate soon falls below the point where the enzyme is fully saturated.

The activity of the hydrogenlyases is expressed as Q_{H_2} (analogous to Warburg's Q_{O_2} for oxygen uptake), that is, it is the number of mm.^{3} of hydrogen evolved by 1 mg. dry weight of bacteria in 1 hour. The samples of the suspensions used were subjected to a nitrogen estimation by the micro-Kjeldahl method. Nitrogen determinations on dried bacteria (washed twice with distilled water) were also made and thus the dry weight of bacteria in any suspension could be arrived at by a nitrogen estimation. The dried bacteria were prepared by drying in a steam-oven to constant weight.

Nitrogen content of dried bacteria.

In order to determine whether the nitrogen content of bacteria varies according to the medium on which they are grown, a series of estimations was made which is summarised in Table I. The medium upon which the cells were grown is indicated in column 2. The broth used was a tryptic digest of caseinogen, containing about 3 % hydrolysed protein. The inorganic medium is that given by Stephenson [1930, p. 275]. Column 3 gives the conditions in which the bacteria were grown. "Roux" indicates that the culture was grown in Roux bottles lying flat, each containing 150 cc. of medium; "flask, aerated" means that the culture was grown in 500 cc. of medium in a litre flask with air bubbling through; "flask, not aerated" means that the preparations were grown similarly, but without air bubbling through.

It will be seen that the effect of the medium or of the growth conditions on the nitrogen content is at most very small. So also is the difference between the various organisms studied. Nicolle and Alilaire [1909], who estimated the nitrogen content of several species of bacteria, found that there

were "différences relativement faibles d'une bactérie à l'autre." Their value for *Bact. coli*, however, was 10·32 % N. They do not state how they dried their bacteria.

Table I. *Nitrogen content of dried bacteria.*

Bacterium	Medium	Conditions	N as % of dry weight
Bact. freundii	Broth formate glycerol	Roux	12·4
Bact. lactis aerogenes (strain 124)	Broth	Roux	13·3
	Broth formate	Roux	12·9
	Broth glucose	Roux	13·6
	Broth formate	Flask, aerated	13·0
	Broth glucose	Flask, not aerated	12·2
	Broth glucose	Flask, aerated	12·0
		Mean	12·8
Bact. dispar	Broth formate	Roux	12·2
	Broth glucose	Roux	13·6
		Mean	12·9
Bact. cloacae (strain 259)	Broth glucose	Roux	12·0
	Inorganic glucose	Roux	11·1
		Mean	11·5
Bact. cloacae (strain 402)	Broth glucose	Roux	13·2
	Broth glucose	Flask, aerated	12·1
	Broth glucose	Flask, aerated	11·9
	Broth glucose	Flask, not aerated	10·3
		Mean	11·9
Bact. coli	Broth	Flask, aerated	12·3
	Broth	Flask, aerated	12·5
	Broth	Flask, not aerated	11·9
	Broth formate	Flask, aerated	12·4
	Broth formate	Flask, aerated	12·0
	Broth formate	Flask, not aerated	12·2
	Broth glucose	Flask, aerated	12·3
	Broth glucose	Flask, not aerated	11·3
		Mean	12·1

Influence of growth conditions on the formation of the hydrogenlyases.

(i) *Bact. coli.* A summary of the results obtained with *Bact. coli* is found in Table II. It is there seen that the production of both hydrogenlyases studied is powerfully influenced by aeration. The condition when the culture is grown on broth formate or broth glucose in flasks is practically anaerobic owing to the rapid evolution of gas and to the small surface exposed. In these conditions the production of hydrogenlyases is at its maximum; with broth formate Q_{H_2} values of 190 and 76 are obtained for formic and glucose hydrogenlyase respectively, while with broth glucose these give Q_{H_2} values of 240 and 185. In Roux bottles, where a large surface is exposed, less enzyme is formed—with broth formate the Q_{H_2} values fall to 52 and 48, and with broth glucose to 215 and 120. With air passing through the medium, no hydrogenlyases are formed, except some glucose hydrogenlyase with broth glucose.

It is seen from Table II that aerobiosis in the formate medium results in an increase in alkalinity. This is explained by the increased oxidation of the formate in aerobic conditions. It might be thought that the decreased amount

Table II. *Hydrogenlyases of* Bact. coli.

Medium	Conditions	Final p_H	Formic hydrogenlyase: mm.³ of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glucose hydrogenlyase: mm.³ of H_2 per mg. N	Glucose hydrogenlyase: Q_{H_2}
Broth	Anaerobic	7·5	290	35	400	48
,,	F.N.A.	7·4	0	0	Small	Small
,,	R.	8·0	0	0	100	12
,,	F.A.	8·3	0	0	0	0
Broth formate	F.N.A.	7·6	1580	190	630	76
,,	R.	8·0	425	52	400	48
,,	F.A.	8·6	0	0	0	0
Broth glucose	F.N.A.	5·4	1970	240	1530	185
,,	R.	5·0	1780	215	980	120
,,	F.A.	5·1	0	0	370	45
Broth glycerol	R.	—	510	62	310	38
Inorg. glucose	F.N.A.	7·0	0	0	0	0
,,	R.	—	0	0	0	0
Inorg. glucose chalk	R.	—	0	0	0	0
Inorg. glycerol	R.	—	0	0	0	0
Inorg. glycerol chalk	R.	—	0	0	0	0

R. = Roux. F.A. = Flask, aerated. F.N.A. = Flask, not aerated.

of the enzymes in these last experiments was due to the concomitant alkalinity. However, aeration in the case of the glucose medium also results in diminished hydrogenlyase activity. Here there is a production of acid, but the different degrees of aerobiosis cause little difference in the final p_H. Increased aerobiosis in this medium again however results in decreased hydrogenlyase activity. It seems, therefore, that the amount of enzyme produced varies inversely with the amount of air present during growth.

Apart from aeration, the formation of formic hydrogenlyase is seen to be most powerfully influenced by the presence of formate. A small amount is formed in strictly anaerobic conditions in broth alone ($Q_{H_2} = 35$) but as formic acid always occurs during the bacterial decomposition of amino-acids, this requires no special explanation. Glucose and glycerol also favour the production of the formic hydrogenlyase, the former to a greater and the latter to a less extent than formate ($Q_{H_2} = 240$ and 62 respectively). Both these substances are, however, known to be decomposed by *Bact. coli* with the formation of formic acid.

Formate also favours the production of glucose hydrogenlyase ($Q_{H_2} = 76$) but not so markedly as glucose itself ($Q_{H_2} = 185$).

Thus formate and glucose in the medium both result in the formation of both hydrogenlyases. The fact that the presence of glucose involves also the presence of formate, accounts for the crossways action in one direction. The production of the glucose enzyme by formate may be due to some characteristic common to the formic and glucose molecules.

The estimation of the Q_{H_2} of the organism grown on an inorganic medium shows that the washed suspensions of such cultures are lacking in both the enzymes. This result was unexpected in view of the fact that hydrogen is formed by *Bact. coli* while growing on an inorganic medium in the presence

of glucose. The reason for this result is not clear. It may be that the absence of the enzyme in the washed suspension is due to the absence of some stabilising substance which is present in the broth.

(ii) *Bact. lactis aerogenes*. The results for two strains of this bacterium are shown in Table III. In calculating the Q_{H_2} values for strain 418, the rate

Table III. *Hydrogenlyases of* Bact. lactis aerogenes.

Medium	Conditions	Final p_H	Formic hydrogenlyase: mm.³ of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glucose hydrogenlyase: mm.³ of H_2 per mg. N	Glucose hydrogenlyase: Q_{H_2}
Strain 124						
Broth	R.	7·5	0	0	0	0
Broth formate	F.N.A.	7·3	2100	270	440	57
,,	R.	—	0	0	0	0
,,	F.A.	7·8	0	0	0	0
Broth glucose	F.N.A.	5·6	Very small	Very small	Very small	Very small
,,	R.	7·1	1800	230	100–440	13–57
,,	R.	—	1600	205	200–610	26–77
,,	F.A.	7·1	17	2	9	1
Inorg. glucose	F.N.A.	6·0	400	50	400	50
,,	R.	6·3	350	45	500	65
Strain 418						
Broth	R.	7·5	0	0	0	0
Broth formate	F.N.A.	7·4	3650	465	500	72
,,	R.	7·6	0	0	0	0
,,	F.A.	7·8	0	0	0	0
Broth glucose	F.N.A.	5·3	0	0	0	0
,,	R.	7·0	4300	550	1400	180
,,	R.	7·1	5500	700	640	82
,,	F.A.	7·1	0	0	400	50
Inorg. glucose	R.	6·0	0	0	1750	225
,,	R.	—	0	0	950	120

R. = Roux. F.A. = Flask, aerated. F.N.A. = Flask, not aerated.

per mg. N was multiplied by the nitrogen content of strain 124. It was found impossible to determine the nitrogen content of strain 418 owing to difficulty in centrifuging. The bacteria came down moderately well when Ringer's solution was used for washing, but when distilled water was used in order to get a preparation of dried bacteria, the cells did not come down even after an hour's centrifuging. Since bacteria of different species have such small differences in their nitrogen content, it is very improbable that there is any appreciable difference between two strains of the same species.

The two strains give exactly similar results. When grown on broth alone, the enzymes are not produced. On broth formate, the bacteria form the enzymes only when little air is present, showing in this respect a greater sensitivity than *Bact. coli*. In Roux bottles, or aerated in a flask, the cultures do not produce the enzymes. The maximum amount of enzyme formed in the case of broth glucose occurs when the culture is grown in moderately aerobic conditions. The very small amounts of the enzymes formed by strain 124, and the absence of the enzymes from strain 418, when the cells are grown in a flask and not aerated, are probably due to the production of acid in these

conditions. The presence of air ensures more complete oxidation of the glucose, and consequently less acid production.

The glucose hydrogenlyase of a suspension of strain 124 which is grown on a broth glucose medium in Roux bottles, shows an initial low output of hydrogen. This increases after about 30 minutes to a new constant rate.

In striking contrast to *Bact. coli*, when grown on an inorganic medium *Bact. lactis aerogenes* produces quite appreciable amounts of the enzymes. Strain 124 forms both hydrogenlyases whilst strain 418 gives only the glucose enzyme. (Both strains produce gas while growing on an inorganic medium with glucose.)

(iii) *Bact. dispar.* This organism produces neither a formic nor a glucose hydrogenlyase no matter on what medium the culture is grown. It does not form gas from formate or glucose in Durham tubes. It is therefore entirely unable to decompose formate or glucose with the liberation of hydrogen, either while growing or when in washed suspension.

(iv) *Bact. cloacae.* Two strains of this species were used. The results are summarised in Table IV.

Table IV. *Hydrogenlyases of* Bact. cloacae.

			Formic hydrogenlyase		Glucose hydrogenlyase	
Medium	Conditions	Final p_H	mm.^{3} of H_2 per mg. N	Q_{H_2}	mm.^{3} of H_2 per mg. N	Q_{H_2}
Strain 259						
Broth	R.	7·1	0	0	0	0
Broth formate	F.N.A.	—	0	0	0	0
,,	R	7·4	0	0	0	0
Broth glucose	F.N.A.	5·5	3450	410	Very small initial	Very small initial
,,	R.	7·1	2900	335	170	20
,,	F.A.	—	Very small	Very small	0	0
Inorg. glucose	R.	6·0	0	0	0	0
,,	F.N.A.	—	0	0	0	0
Strain 402						
Broth	R.	7·1	0	0	0	0
Broth formate	F.N.A.	7·0	0	0	0	0
,,	R.	7·4	0	0	0	0
Broth glucose	F.N.A.	5·5	1560	200	1610	190
,,	R.	7·4	3100	370	200	24
,,	F.A.	7·3	170	20	395	47
Inorg. glucose	R.	—	0	0	0	0
,,	F.N.A.	—	0	0	0	0

R. = Roux. F.A. = Flask, aerated. F.N.A. = Flask, not aerated.

Broth alone or broth formate give no hydrogenlyases in whatever conditions growth takes place. The only medium which results in the formation of the enzymes is broth glucose. The formic hydrogenlyase of strain 259 is best formed in the presence of little air. Strong aerobiosis almost completely inhibits its formation. The glucose enzyme on the other hand is most developed in moderately aerobic conditions. In the case of strain 402, the converse occurs. Here it is the formic enzyme which is at its maximum in

moderately aerobic conditions, and the glucose enzyme which is best formed when little air is present.

No enzymes are formed on an inorganic medium, although, as with *Bact. coli* and *Bact. lactis aerogenes*, gas is formed during growth on such a medium containing glucose.

(v) *Bact. freundii*. This is an organism of the coli-typhosus group. It was first described by Braak [1928]. Its chief biochemical interest lies in the fact that it is able to grow anaerobically in an inorganic medium with glycerol as sole carbon source. This is attributed to its power of oxidising one molecule of glycerol at the expense of a second, which is simultaneously reduced to trimethyleneglycol.

Table V gives the results of a number of experiments undertaken to discover what media are necessary for the production of the hydrogenlyases by this organism. In all cases except those specifically mentioned, growth was allowed to take place for 15–18 hours in Roux bottles.

Table V. *Hydrogenlyases of* Bact. freundii.

Exp. No.	Medium	Final p_H	Formic hydrogenlyase: mm.³ of H_2 per mg. N	Formic hydrogenlyase: Q_{H_2}	Glycerol hydrogenlyase: mm.³ of H_2 per mg. N	Glycerol hydrogenlyase: Q_{H_2}
1	Broth	7·3	1710	210	330	40
2	,,	7·3	2100	260	600	75
3	Broth formate	7·8	3500	430	—	—
4	,,	8·0	7400	920	690	85
5	,,	—	4300	535	290	36
6	Broth formate glycerol	7·3	3020	375	900	110
7	,,	7·4	2700	335	750	93
8	,,	7·3	3600	445	1350	170
9	,,	7·4	2900	360	1020	125
10	,,	7·3	—	—	1600	200
11	,,	—	2450	305	1000	125
12	Broth glucose	—	410	50	(240	30)
13	Inorg. glycerol (aer.)	—	0	0	0	0
14	,, (anaer.)	—	0	0	0	0
15	,, chalk (aer.)	—	0	0	0	0
16	,, ,, (anaer.)	—	0	0	0	0
17	Inorg. glucose (aer.)	—	0	0	0	0
18	,, chalk (aer.)	—	340	42	(340	42)
19	Inorg. acetate	—	0	0	0	0
*20	Inorg. broth glycerol	6·8	230	28	140	17
21	,,	—	4000	495	2000	250
22	Inorg. gelatin glycerol	—	0	0	0	0
23	Inorg. albumin glycerol	—	+	+	+	+
24	Inorg. amino-acids glycerol	—	0	0	0	0
25	Broth nitrate	—	0	0	0	0
26	Broth nitrate formate	6·8	240	30	0	0

* Exp. 20 was made with diluted broth.

Exps. 13–18 were all repeated several times. The only positive result—that of Exp. 18—was obtained by growing the culture for 40 hours. An 18-hour culture possessed no lyases.

In Exps. 12 and 18 glucose hydrogenlyase was investigated instead of the glycerol hydrogenlyase.

The media, broth and inorganic, were always diluted 1:3 from stock media. The medium in Exp. 20 contained 10 % of the undiluted broth and 33 % of the undiluted inorganic medium. That in Exp. 21 contained 33 % undiluted broth and 33 % undiluted inorganic medium; that is, it contained the normal amount of broth, as used in Exps. 1–12.

It will be seen that the values of the Q_{H_2} for bacteria grown on the same medium, and acting on the same substrate vary considerably. However, the ratio of the values for the different substrates acted upon by bacteria grown on the same medium is fairly constant. The ratio of the rates of action on glycerol and on formate has also been found in a number of cases where the Q_{H_2} is not known, the nitrogen value of the suspension not having been determined.

Bact. freundii grown on broth alone aerobically acts on glycerol at about one-quarter of the rate at which it acts on formate. Three determinations (Exps. 1 and 2, and another in which the nitrogen was not estimated) give 19, 28 and 25 %—average 24 %. Grown on broth anaerobically, the ratio is 15 %. When formate is present, the rate for glycerol remains about the same as on broth alone aerobically, whilst the rate for formate is approximately doubled. The ratios Q_{H_2} for glycerol/Q_{H_2} for formate in four experiments are 9, 7, 6 and 10 %—average 8 %. Grown on broth glycerol, suspensions give the ratios 63, 67 and 72 %—average 67 %. If both glycerol and formate are present, the ratio is 34 %.

We see then that the addition of formate to the medium increases the amount of formic hydrogenlyase produced, whilst the addition of glycerol increases both. A mixture of formate and glycerol gives a proportion of glycerol hydrogenlyase intermediate between those obtained in the presence of formate or glycerol alone.

Considering the results in Exps. 1–12 alone, one would have to say that, according to Karström's definition of constitutive and adaptive enzymes, the hydrogenlyases of both formic acid and glycerol are constitutive, since they are found when the organism is grown in the absence of these substrates. There are, it is true, small amounts of formic acid produced in the broth, and a similar suggestion may be made with regard to the presence of the hydrogenlyases in *Bact. freundii* when grown in broth alone as was made in the case of *Bact. coli* grown anaerobically on broth. One would then assume that *Bact. freundii* is more sensitive to the presence of formic acid than is *Bact. coli*.

The results of Exps. 13–24 show that only in very special conditions are the enzymes formed on a medium which does not contain broth. When the organism is grown on an inorganic medium, the enzymes are almost invariably absent. Small amounts of the formate and glucose hydrogenlyases are formed when the cells are grown in the presence of chalk, on a medium containing glucose. On a medium containing glycerol, aerobically or anaerobically, in the presence or absence of chalk, the hydrogenlyases are never produced. The non-formation of the enzymes when the culture is made in an inorganic

medium is not due to an inhibitory substance present in this. Exp. 21 shows that if the normal amount of broth is present, the amount of enzyme produced is the same as if no inorganic medium is present. If less broth is present (Exp. 20), a proportionately smaller amount of hydrogenlyase is formed.

It was thought that the absence of the hydrogenlyases when *Bact. freundii* is grown on an inorganic medium might be due to a destruction of the enzymes by the acid formed from the glycerol. However, when a large amount of phosphate buffer was added to the inorganic glycerol medium, no enzymes were formed. This was not due to a poisoning action of the buffer, since a similar amount of buffer added to broth medium resulted in no reduction of the hydrogenlyase activity.

Bact. freundii produces gas whilst growing on an inorganic medium + glucose.

Some experiments were performed in order to investigate the effect of formate in an inorganic medium. It was found, however, that the presence of formate entirely inhibited growth in several media in which growth otherwise occurs.

Unhydrolysed protein is not sufficient to ensure the production of any appreciable amount of enzyme (Exps. 22, 23). 0·5 % gelatin in the medium gives no hydrogenlyases at all. The presence of 0·5 % albumin gives a very small amount of enzyme. The Q_{H_2} was not measured since some of the protein was precipitated and consequently the nitrogen value did not give a true measure of the amount of bacteria. However, a very thick suspension was used, and the rate of hydrogen output was only just measurable.

A mixture of amino-acids was then tried. The medium contained about 1 % of a mixture of glycine, alanine, leucine, tyrosine, tryptophan, histidine, glutamic acid and aspartic acid, together with inorganic medium and 0·5 % glycerol. The suspension of bacteria which had been grown on this medium did not possess the hydrogenlyases.

It will be seen from Table V that nitrate entirely inhibits the production of the enzymes from broth alone. The addition of nitrate to a broth formate medium results in the formation of a small amount of the formic hydrogenlyase but of no glycerol hydrogenlyase.

Discussion.

Hydrogenlyases are formed by many species of bacteria in certain conditions. The factors influencing their formation are seen to vary somewhat with the organism studied, but the following general conclusions may be drawn.

Aeration has an inhibitory effect on the formation of the hydrogenlyases. This is not due to a destruction of the enzymes. Once the enzymes have been formed, aeration of a washed suspension of the bacteria, in which the enzymes are present, does not destroy them.

Suspensions of the two strains of *Bact. lactis aerogenes*, and *Bact. cloacae*, strain 259, were aerated for 24 hours at room temperature. For the same

period, portions of the same preparations were left in a vacuum, also at room temperature. The activities of the hydrogenlyases were determined before and after the experiment (Table VI).

Table VI. *Effect of aeration on activity of hydrogenlyases.*

	Q_{H_2} (formic hydrogenlyase)			Q_{H_2} (glucose hydrogenlyase)		
Bacterium	Initial	After vacuum 24 hrs.	After aeration 24 hrs.	Initial	After vacuum 24 hrs.	After aeration 24 hrs.
Bact. lactis aerogenes						
Strain 124	225	225	200	35	100	73
Strain 418	250	250	270	35	130	50
Bact. cloacae						
Strain 259	510	435	375	75	175	75

The formic hydrogenlyases are at most slightly decreased either by keeping in a vacuum or by aeration. The effect on the glucose enzyme of keeping in a vacuum is to increase it considerably. Aeration, on the other hand, results in rather less increase.

It may be concluded, therefore, that aeration of a growing culture does not result in the destruction of formed enzymes but actually prevents their formation. This may possibly be due to the oxidation of some substance in the medium necessary for the production of the enzymes.

A second factor is the presence of broth. Although all the organisms except *Bact. dispar* form hydrogen from glucose while growing in an inorganic medium, only *Bact. lactis aerogenes* and, in very special conditions, *Bact. freundii* give the enzymes in washed suspension of cultures from this medium. It is possible that the enzymes in these conditions are less stable, or formed in extremely small amounts, and are thus found only in some cases. The problem is being further investigated.

A third factor is the presence of the substrate. The influence of the substrate on the production of organisms possessing the hydrogenlyases may be pictured as either (1) a natural selection, or (2) a chemical adaptation. In the former case, one must suppose that there exists in all cultures a small but definite number of cells possessing the enzyme. Since at some period each of the organisms used has been grown from a single cell colony, one must imagine a biochemical variation or mutation of a definite though low frequency, in order to account for the existence of the cells containing the hydrogenlyases. Such variations have been known to occur. Neisser [1906] described the sudden appearance in a certain coliform bacterium of an ability to ferment lactose, and Massini [1907] showed that the new strain was immunologically identical with the old strain. Enzymes, then, arising by some such mutation, become, according to this hypothesis of natural selection, of biological value to the organism. Those members possessing them are therefore at an advantage and tend to multiply at the expense of the others. A strain is thus formed in which the majority of the members possess the enzyme in question.

Such an explanation presupposes that the reaction catalysed by the enzyme is of service to the organism. In the case of the formic hydrogenlyase this is difficult to demonstrate. The reaction $HCOOH = H_2 + CO_2$ can hardly be of any value to the cell. The heats of reaction show that it is only slightly exothermic, and although the data for the free energy change are not known, it is probable that little or no energy is available to the cell. Moreover, the end products cannot enter into the cell structure. It is of course possible that formate is detrimental to the organism and that the action of the hydrogenlyase permits of its removal anaerobically. Such an explanation is supported by the following facts: (1) the hydrogenlyases are not formed in highly aerobic conditions, where the removal of formate occurs by the action of formic dehydrogenase, and (2) in inorganic media where growth normally takes place, sodium formate is entirely inhibitory (see above *Bact. freundii*).

On the other hand, it is difficult to see how this applies to the cases of glucose and glycerol.

Against the selection hypothesis the following experiment may be quoted. *Bact. freundii* was grown for a month, with frequent sub-culturing, on broth glycerol in one case, and on broth formate in another. After this time, an inoculation was made from each of these into plain broth, and the Q_{H_2} values for formate and glycerol determined in each case after the usual 15 hours' growth. It was found that these were of exactly the same order as was obtained when the inoculation was made into plain broth from "untrained" *Bact. freundii*.

Hence, if selection had occurred during cultivation on the glycerol or formate medium, the character acquired was immediately lost on returning to plain broth. This must again involve natural selection of the few remaining unaltered cells. This presupposes that the loss of the hydrogenlyases is an advantage when the cells are growing in plain broth—a supposition difficult to support.

More plausible is the second suggestion, that of chemical adaptation. On this view, an adaptive enzyme arises as a response to its chemical environment. It would then partake of the nature of an acquired character in higher organisms. With the removal of the stimulus, the character is lost by the descendants of the organism. They still, of course, retain the power to develop that character in conditions similar to those in which the parent organism developed it. This explanation, though admittedly incomplete, is in accordance with the experimental facts so far determined.

In this way, one would distinguish between "training" and "adaptation" by considering the former to be an inheritable variation, whilst the latter is a specific response to a change in the environment and is hence of the nature of an uninheritable acquired character.

SUMMARY.

1. The conditions governing the formation of the hydrogenlyases in several species of bacteria have been studied.

2. A method for measuring the activity of the culture has been worked out. The unit employed is the Q_{H_2}, *i.e.* mm.^{3} of hydrogen liberated per mg. of dry bacteria per hour.

3. Aerobic conditions operating during growth prevent the formation of the enzymes in some cases and greatly decrease their production in others.

4. Aerating a washed suspension does not destroy the enzyme. It is therefore the actual formation of the enzyme which is inhibited by air.

5. In most cases, no hydrogenlyases can be demonstrated in organisms grown on a simple inorganic medium, whereas organisms grown in presence of broth contain them.

6. The presence in the growing culture of the substrates on which the enzymes act is either a necessary factor in their production or very greatly increases the amount formed.

7. The formation of a culture which possesses the hydrogenlyases is probably the result of a specific action on the growing organisms of certain substances in the medium in which they are grown. It is unlikely that a natural selection of pre-existing enzyme-containing organisms occurs.

8. A distinction is drawn between "training" and "adaptation." The former seems to be a selection of a variation, the latter a direct response to the stimulus of the chemical environment.

I am glad to take this opportunity of expressing my gratitude to Miss Marjory Stephenson at whose suggestion this work was undertaken and whose advice and constant encouragement were invaluable. My thanks are also due to Sir F. G. Hopkins for his interest in this work.

REFERENCES.

Abderhalden (1925). *Fermentforsch.* **8**, 42.
Braak (1928). Onderzoekingen over vergisting van glycerine. Diss. Delft.
Dienert (1900). *Ann. Inst. Past.* **14**, 139.
Dubourg (1899). *Compt. Rend. Acad. Sci.* **128**, 440.
Harden and Norris (1910). *Proc. Roy. Soc. Lond.* B **82**, 645.
Karström (1930). Thesis. Helsingfors.
Klöcker (1900). *Medd. Carlsberg Lab.* **5**, 55.
Klotz (1906). *J. Infect. Dis.* **2**, 35.
Massini (1907). *Arch. Hygiene*, **61**, 250.
Neisser (1906). *Zentr. Bact. Par.* I. Ref. **38**, 98 (Beiheft).
Nicolle and Alilaire (1909). *Ann. Inst. Past.* **23**, 547.
Stephenson (1930). Bacterial metabolism. (London, Longmans, Green and Co.)
—— and Stickland (1932). *Biochem. J.* **26**, 712.
Twort (1907). *Proc. Roy. Soc. Lond.* B **79**, 329.
Willstätter and Sobotka (1922). *Z. physiol. Chem.* **123**, 176.

CCXXII. PYRUVIC ACID AND VITAMIN B_1 DEFICIENCY.

By ARNOLD PETER MEIKLEJOHN, REGINALD PASSMORE and RUDOLPH ALBERT PETERS.

From the Biochemistry Department, Oxford.

(*Received September 24th, 1932.*)

The significance of pyruvic acid in carbohydrate metabolism of animal tissues has long been discussed. Since Meyerhof, Lohmann and Meier [1925] showed that out of a large number of substances investigated only pyruvic acid and lactic acid increased the oxygen uptake and carbohydrate content of muscle, pyruvic acid has been suggested as a stage in the oxidation or oxidative re-synthesis of lactic acid. Only recently, however, have Case and Cook [1931] been able to detect pyruvic acid in muscle in the absence of fixative under both aerobic and anaerobic conditions. A discussion of the possible origin and fate of this pyruvic acid is given in a recent paper by Case [1932, 2].

We have shown [Gavrilescu and the authors, 1932] that the action of vitamin B_1 concentrates *in vitro* was specifically related to the lactic oxidase system of pigeon's brain; no evidence could be obtained of interaction with the succinoxidase system. A desire to settle the point of action of the vitamin concentrates led us to the study of the behaviour of normal and avitaminous pigeons' brains in the presence of pyruvic acid. The experiments were made more interesting by the realisation that light might be thrown upon the fundamental problem of the rôle of pyruvate in carbohydrate metabolism.

Our work has elicited the unexpected fact that the oxygen uptake in the presence of pyruvate is always much more depressed than with lactate in the avitaminous brain; further, contrary to experience with lactate, this depression is not correspondingly improved by the addition of vitamin B_1 concentrates *in vitro*. Normal brains show good oxygen uptakes with pyruvate as substrate. We have not been able to establish a satisfactory explanation of these facts.

Experimental.

Method. The oxygen uptake of minced pigeon's brain has been determined as previously described [Gavrilescu *et al.*, 1932]. The estimations have all been done at 38° with 50–100 mg. of minced tissue suspended in 3·0 cc. of phosphate buffer solution. The vitamin B_1 concentrate had an activity of approximately 0·05 mg. for the bird and was the same as previously used. Vitamin B_1 deficiency was induced in the birds by our usual methods.

Pyruvic acid. The literature dealing with pyruvic acid in relation to oxidase and other biological systems is singularly silent upon the methods used to ensure the purity of the acid under experiment[1]. For our purposes freedom from the last traces of lactic acid, and of possible toxic impurities (see below) was important. We have therefore relied upon one or two redistillations of a pure commercial sample (Messrs Boots and Co.) and have in many cases used freshly distilled acid. Our samples did not crystallise, but distilled within a close range of 0·5–1·0° at pressures of 15–20 mm. Hg and were quite colourless. After keeping for 1 month the titration of one sample gave 93 % —COOH and 88 % by iodine value. Specimens of the 2:4-dinitrophenylhydrazone prepared direct from aqueous solutions gave the correct melting-point, and we have little doubt that a sufficient approach to purity was made for our purposes.

Results.

Oxygen uptake in the presence of varying concentrations of pyruvate.

Table I shows the oxygen uptake of normal and avitaminous cerebrums and optic lobes/rest in the presence of varying concentrations of pyruvate. Fig. 1 shows typical curves for normal and avitaminous cerebrums. The

Table I. *Effect of varying concentrations of pyruvate.*

Oxygen uptake in mm.³/g./hr.

Exp.	Ringer	Lactate	Pyruvate 0·007 *M*	Pyruvate 0·017 *M*	Pyruvate 0·034 *M*	Pyruvate 0·102 *M*
			Normal brains.			
			(*a*) Cerebrums.			
264	(850)	—	1750	1930	1705	(1540)
276	770	2150	2040	2120	2230	2000
278	740	1960	1980	(2350)	2300	(2050)
279	1005	1860	1710	1680	1935	1575
			(*b*) Optic lobes/rest.			
264	510	—	—	—	(1300)	1200
286	635	—	1830	2005	—	—
			Avitaminous brains.			
			(*a*) Cerebrums.			
262	775	—	1085	970	925	735
265	740	—	1010	(1080)	975	895
			(*b*) Optic lobes/rest.			
265	(660)	—	875	(870)	790	—

apparent Michaelis constant for the enzyme systems under these conditions is of the order of 0·003 *M*, both for normal and avitaminous brains. This is approximately the same as the constant previously obtained for lactate under similar conditions, if we allow for the presence of inactive lactate in the racemic mixture.

[1] Since the completion of this work, the papers by Case [1932, 1, 2] have appeared. The paper by Wendel [1932] also appeared while this was in progress.

A factor, however, appears which was not present in our previous experiments with succinate and lactate, namely inhibition with higher concentrations. This effect was obtained with our most highly purified specimens, and in our opinion cannot be due to a toxic impurity. Bernheim [1928] showed that pyruvic acid in the same concentrations had an inhibitory effect on the lactic acid dehydrases of yeast. We have found no evidence of pyruvic acid inhibiting the oxygen uptake in the presence of lactate at concentrations below 0·034 *M*. As a result of this inhibitory effect there is no broad optimum for maximum oxygen uptakes. In one experiment the maximum was at 0·007 *M*, and the inhibition started at 0·017 *M*. In another there was no inhibition with 0·03 *M* pyruvic acid. We have worked with 0·007 *M* and 0·034 *M* pyruvate, and although we cannot be certain that these will give maximum uptakes, they will not be far from the optimum.

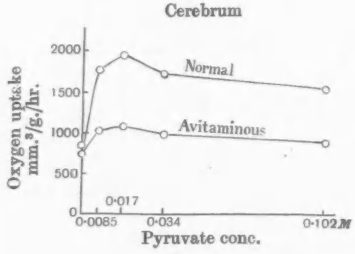


Fig. 1.

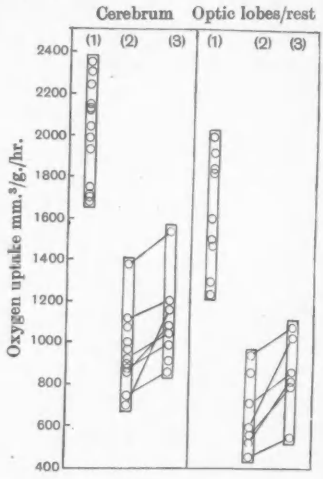


Fig. 2.

Fig. 1. Oxygen uptake of cerebrum from pigeon, normal and avitaminous, in relation to concentration of pyruvate.

Fig. 2. Oxygen uptake of pigeon's brains, (1) normal, (2) avitaminous, (3) avitaminous + vitamin B_1 concentrate.

Oxygen uptake for normal and avitaminous brains.

(1) *Comparisons with lactate and pyruvate as substrates.* Fig. 2 shows all the results obtained with normals using pyruvate as substrate. Most of the figures are in Table I and the averages are in Table II. Table III contains all the results for avitaminous brains and the effects of addition of vitamin B_1 concentrates *in vitro*.

It will be seen that the normal brains take up as much oxygen in the presence of pyruvic acid as in the presence of lactic: indeed the averages are

Table II. *Pyruvate results.*

Normals.

Oxygen uptake in mm.³/g./hr.

	Lactate	Pyruvate 0·007 *M*	Pyruvate 0·017 *M*	Pyruvate 0·034 *M*
Average cerebrum	1620	1870	2010	1895
Average optic lobes/rest	1380	1840	1800	1480

Table III.

Oxygen uptake in mm.³/g./hr.

Exp.	Lactate	Pyruvate Concentration *M*	Pyruvate Control	Pyruvate Vitamin	Increase %
		Avitaminous birds.			
		(*a*) Cerebrums.			
238	—	0·034	730	870	19
241	(1360)	0·034	900	995	10
245	1160	0·034	705	(1165)	66
249	1375	0·034	890	915	2
266	—	0·034	900	1050	17
300	1430	0·017	1380	(1550)	12
301	1290	0·017	(1340)	1600	19
266	—	0·017	885	1090	23
262	—	0·017	970	—	—
265	—	0·017	(1080)	—	—
267	1200	0·007	920	1210	30
281	(1150)	0·007	(1125)	1210	8
262	—	0·007	1085	—	—
265	—	0·007	1010	—	—
		Average	990	1160	20
		(*b*) Optic lobes/rest.			
245	—	0·034	600	(1040)	70
248	—	0·034	460	560	22
249	—	0·034	570	810	42
262	—	0·034	530	830	57
300	1130	0·017	(955)	8090	14
301	(980)	0·017	770	1140	48
280	—	0·007	720	875	21
265	—	0·007	875	—	—
		Average	610	910	40

higher. On the other hand the uptake of the avitaminous brains is very much lower than for lactate, *e.g.* Exp. 245; residual oxygen uptake 665; pyruvate 705; lactate 1160. It might be considered from these results that depressed oxidation with pyruvate represents a more fundamental lesion than that with lactate. The effect of addition of vitamin B_1 concentrates, however, does not confirm this.

(2) *Addition of vitamin B_1 concentrates.* Reference to Table III and Fig. 2 shows that the effect of the concentrate is very variable. Often the increase can be entirely explained by the effect on the residual uptake. Often, especially in the optic lobes/rest, the effects are large, but in no case does the concentrate ever raise the uptake above 1140 mm.³/g./hr., where the minimum normal

figure is 1550, and the average 1850. The vitamin acting *in vitro* is unable to restore the oxidising powers to anything approaching normal with pyruvate as substrate. This suggests that the change is not connected with the deficiency of vitamin B_1 directly. If this interpretation of the results is correct, it raises two possibilities. The failure of the avitaminous brain to take up oxygen in the presence of pyruvic acid might be due either to changes in the brain tissue secondary to the vitamin B_1 deficiency, or to the absence from the avitaminous minced brain of some necessary somatic factor (supplied by the blood stream).

Birds killed with iodoacetic acid.

With regard to the first possibility, the only change of which we know, apart from changes in oxidation, is the increase in lactate in avitaminous as compared with normal brains. If lactate and pyruvate were both oxidised at some common centre, it might be that the increased concentration of lactate blocked the pyruvate. This we have tried to test by killing birds with iodoacetate (IAA), which is known to inhibit lactic acid production in these brains. Kinnersley and Peters [1930] for instance found in normals killed with IAA 20–30 mg./100 g. of lactic acid and in avitaminous 30–60 mg.[1] This would be quite different from the normal 100–150 mg./100 g. and avitaminous 150–250 mg./100 g. The dose of IAA used for killing the birds was 75 mg./1000 g. body weight. For safety we have used IAA in the bottles during the experiments in a concentration 0·1 mg. per 1 cc. of Ringer as suggested by Fisher [1931]. This has some effect on oxidation both with pyruvic acid and lactic acid, as the poison is not absolutely defined in its action [Lohmann, 1931], and with increased concentrations glyoxylase systems are affected [Dudley, 1931]. The decrease is shown in Table IV. With normal brains the pyruvate and lactate

Table IV. *Iodoacetic acid experiments.*

Oxygen uptake in mm.³/g./hr.

	Cerebrums			Optic lobes/rest		
Exp.	Ringer	Lactate	Pyruvate	Ringer	Lactate	Pyruvate
			(*a*) *Normal brains.*			
289	460	(1150)	1135	380	1125	(1120)
293	(440)	(1380)	1300	385	945	1100
296	550	1610	1165	515	1405	1095
299	500	1390	1240	(425)	1060	1070
			(*b*) *Avitaminous brains.*			
288	490	(1030)	(775)	—	560	(510)
294	505	970	(670)	—	650	450
297	530	1160	900	320	780	550
298	430	1000	670	390	750	580

oxygen uptakes are almost identical. The extra oxygen uptake for pyruvate has disappeared. In one experiment, No. 296, however, the pyruvate oxidations are much more markedly depressed than the lactate. There is an indication that the IAA has a possible specific inhibitory effect on pyruvic acid

[1] The cerebrum generally gave the lower figures.

oxidations, and this must moderate any conclusions drawn from these experiments. In the avitaminous brains there is still the increased depression of oxygen uptake in the presence of pyruvate and there is no marked improvement on vitamin addition. Even with the lowered lactic acid content of the IAA brains the pyruvate is less efficient. Hence there is no indication that the changes in pyruvate behaviour are due to blockage by lactate.

The oxygen uptake in the presence of pyruvic acid of the brains of birds cured by dosing with concentrates.

The authors [1932] had previously investigated the oxygen uptake of cured avitaminous birds' brains in the presence of lactate. Table V gives figures for pyruvate and lactate for two groups of birds both dosed with 8–12 pigeon

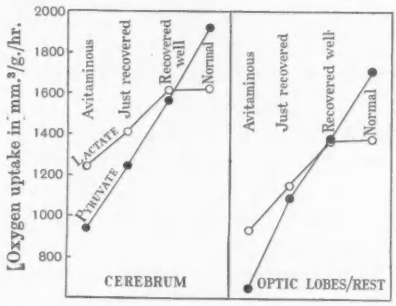


Fig. 3. Chart showing oxygen uptake (average) in relation to condition of bird, avitaminous, just recovered, recovered well, normal. ● pyruvate, ○ lactate as substrate.

Table V. *Dosed birds.*

Oxygen uptake in mm.³/g./hr.

	Cerebrums				Optic lobes/rest	
	Lactate	Pyruvate			Lactate	Pyruvate
Exp.		Concentration *M*	Control	Vitamin		
		(*a*) *Birds just out of symptoms.*				
291	1430	0·017	(1200)	(1235)	995	890
290	1325	0·017	1280	(1290)	1395	1085
274	1350	0·007	1320	1790	1225	1320
284	1380	0·017	(1220)	(1270)	1270	1040
273	1520	0·007	(1355)	1600	(1220)	1100
285	(1430)	0·017	(1100)	1510	1005	1005
Average	1410		1245	1450	1140	1070
		(*b*) *Birds well recovered.*				
283	1665	0·017	1580	(1770)	(1380)	(1410)
292	1540	0·017	1500	—	(1295)	(1285)
272	(1480)	0·007	1405	(1570)	(1350)	—
277	1685	0·007	(1730)	1720	1420	—
282	1660	0·017	1780	1600	1380	(1475)
275	1650	0·007	(1400)	1790	1405	1270
Average	1615		1565	1690	1370	1380

doses (approximately vitamin B_1 units). Group (*a*) had only just recovered from head retraction and were very weak. Group (*b*) had had a longer time for recovery. The averages for lactate and pyruvate for each group and for normals and avitaminous are shown in Fig. 3. There is a recovery of oxygen uptake in the presence of pyruvate, but it is not so complete as for lactate. No relationship has been found between the degree of recovery with the two substrates in individual birds. We feel that these experiments indicate that the fundamental lesion associated with the nervous symptoms is concerned with the lactate, and that the change in behaviour to pyruvate is in some way secondary.

Discussion.

These experiments make it difficult to believe that pyruvic acid is a normal stage in oxidation of lactic acid, unless it be assumed, as suggested by Neuberg [1927] that pyruvic acid formed in the tissues molecule by molecule is constitutionally different from added pyruvic acid. In this respect, we are in agreement with Case and Cook [1931] and Case [1932, 1, 2].

We have thought that for the normal oxygen uptake of pyruvate to be regained by avitaminous tissue a somatic factor might be required, whose co-operation with the vitamin is needed for normal oxidations in the presence of pyruvic acid. We cannot say whether such a factor is needed to remove a toxic substance in the tissues or to provide a co-ferment. A few experiments with addition of serum have given very varied and inconclusive results.

Summary.

1. Normal pigeon's brain when minced gives a large oxygen uptake in the presence of pyruvic acid.

2. The avitaminous pigeon's brain gives a very low oxygen uptake in the presence of pyruvic acid, which is not restored by addition of vitamin B_1 concentrate *in vitro*. These same concentrates are capable of restoring lowered oxygen uptake with lactate as substrate.

3. The defect in oxidation is not associated with the high lactic acid content of the avitaminous brains.

4. There is no indication that pyruvic acid is a stage in the oxidation of lactic acid in the pigeon's brain.

We are indebted to Mr H. W. Kinnersley for the preparation of the concentrates used in these experiments, also to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants towards the cost of the experiments.

REFERENCES.

Bernheim (1928). *Biochem. J.* **22**, 1178.
Case (1932, 1). *Biochem. J.* **26**, 753.
—— (1932, 2). *Biochem. J.* **26**, 759.
—— and Cook (1931). *Biochem. J.* **26**, 1318.
Dudley (1931). *Biochem. J.* **25**, 439.
Fisher (1931). *Biochem. J.* **25**, 1411.
Gavrilescu, Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **110**, 431.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 710.
Lohmann (1931). *Biochem. Z.* **236**, 444.
Meiklejohn, Passmore and Peters (1932). *Proc. Roy. Soc. Lond.* B **111**, 391.
Meyerhof, Lohmann and Meier (1925). *Biochem. Z.* **157**, 459.
Neuberg (1927). *Biochem. Z.* **180**, 471.
Wendel (1932). *J. Biol. Chem.* **94**, 717.

CCXXIII. THE INFLUENCE OF THE ADMINISTRATION OF ARGININE AND HISTIDINE ON THE EXCRETION OF CREATININE.

By BENNIE G. SHAPIRO and HARRY ZWARENSTEIN.

From the Department of Physiology, University of Cape Town.

(*Received September 28th, 1932.*)

The numerous attempts to demonstrate the origin of muscle-creatine or urinary creatinine from arginine or histidine have been almost entirely negative in result or open to serious criticism. The fact that both arginine and histidine are subject to destruction by the liver has no doubt militated against any positive result in the majority of cases, especially when small amounts of the amino-acids have been administered *per os*.

In regard to histidine more definite evidence has recently been presented by Abderhalden and Buadze [1929; 1930], who have shown that feeding large amounts of histidine or haemoglobin led to significant increases in creatinine excretion.

Reviews of the literature dealing with the effect of administering arginine, histidine, cystine and other substances are given by Hunter [1928], Hyde and Rose [1929] and Abderhalden and Buadze [1929]. In a recent paper Beard and Barnes [1931] reported the effects of administration of protein, amino-acids and related substances on muscle-creatine and urinary creatinine in adult rats and also the effects of these substances on creatinine elimination in man. A large number of amino-acids, including histidine, arginine, glycine, glutamic acid, tyrosine and cystine, the proteins edestin and caseinogen, and finally choline and glycocyamine were found to increase muscle-creatine. Negative results were obtained with sarcosine, nucleic acid, betaine and taurine. All the amino-acids and proteins studied increased the elimination of creatinine.

These authors believe that their results cannot be ascribed to the effect of specific dynamic action and conclude that the creatine formation and creatinine excretion were due either to feeding large quantities of proteins and amino-acids or to the possibility that these substances stimulated creatine-creatinine metabolism in some other way than by specific dynamic action. They favour the former view but taking into account the large number and variety of substances giving positive results a more likely explanation is one based on the assumption of a generalised stimulation of creatine-creatinine metabolism. However, several investigators [Lewis, Dunn and Doisy, 1918;

Zwarenstein, 1928; Christman and Mosier, 1929] have shown that administration of several amino-acids, including glycine which gave the maximum effect in Beard and Barnes' experiments, had no effect on creatinine excretion. A satisfactory explanation of the above results is difficult and the findings of Beard and Barnes are in obvious need of re-examination.

In the work described below attention has been focused largely on the effects of arginine and histidine but some of the experiments of Beard and Barnes with other amino-acids have been repeated. During the course of research on other lines in this laboratory it was found that rabbits were very suitable animals for investigations on creatine-creatinine metabolism. The daily output of creatinine is constant within comparatively narrow limits except for slight seasonal variations and is furthermore independent of caging or of the usual diet of carrots, cabbages and bran. For short period experiments such as those described below the normal daily creatinine excretion remains remarkably constant.

Experimental.

The experiments were carried out on adult male rabbits. Details in regard to caging, feeding and collection of urine have been described elsewhere [Schrire and Zwarenstein, 1932]. Creatine and creatinine were estimated by Folin's method. Eight animals were used in all and before the effects of the various substances were investigated the daily creatinine excretion was estimated for periods of 4 or 5 days over several weeks and thus a normal preperiod consisting of about 18 daily creatinine figures was obtained. On the day preceding administration of the substance creatinine was again determined thus checking any large deviation from the normal.

The histidine hydrochloride, arginine, glycine and alanine were dissolved in Locke's solution (30–35 cc.) and the solutions of the first two were neutralised with dilute NaOH or HCl before injection. Cystine, tyrosine, glutamic acid and glycocyamine were fed mixed with bran. Only in the case of cystine did the animals fail to ingest all the mixture, so that the amounts for cystine in Table II are actually rather less than stated. In some experiments the animals received a single injection, in the majority of experiments they were injected or fed once a day on three consecutive days. Four animals were usually put into separate metabolism cages at the same time. One of these always received Locke's solution only or bran and served as a control. Every animal in its turn served as a normal control in the various experiments. In all cases the diet consisted of cabbages and carrots. The controls for the feeding experiments were given bran in addition. For the sake of convenience the data in the following Tables are arranged in a slightly different sequence from that in which they were actually obtained. Injection of 35 cc. of Locke's solution daily for 3 days had no effect on the excretion of creatinine. Similarly, the addition of bran to the usual ration had no effect. Small quantities (10 to 50 mg.) of the different amino-acids were added to 10 cc. portions of a filtered

urine, and the creatinine was estimated and compared with the value obtained from the same urine without the addition of the amino-acid. The readings were identical in all cases except on addition of alanine or cystine and for these two substances the differences were not significant.

The figures in the Tables below unless otherwise stated refer to mg. creatinine excreted in 24 hours. The figures in brackets refer to the amount of the substance in g. fed or injected.

Table I.

Rabbit No. ...	I	II	III	IV
Weight (kg.) ...	2·46	2·52	2·18	2·03
A. *Pre-period*				
No. of 24 hour periods	18	16	18	17
	96 ±2	98 ±5	90 ±6	92 ±5
B. *Arginine*				
Preceding day	100	101	90	95
Injection period (1 day)	109 (5)	136 (5)	106 (5)	89 (2·5)
	103	101	103	99
	107	93	93	103
	102	110	80	99
Preceding day	—	—	—	91
Injection periods (3 days)	—	—	—	91 (2·5)
	—	—	—	114 (3·0)
	—	—	—	114 (5·0)
C. *Histidine*				
Preceding day	99	106	84	92
Injection period (1 day)	94 (3)	138 (5)	104 (3·5)	109 (3·5)
	100	126	96	96
	110	109	85	80
	103	113	—	—
Preceding day	104	—	93	—
Injection periods (3 days)	125 (2·5)	—	93 (2·5)	—
	96 (3·5)	—	96 (3·0)	—
	117 (4·0)	—	120 (5·0)	—

Table II.

Rabbit No. ...	III	IV	V	VI
Weight (kg.) ...	2·18	2·03	1·80	1·77
A. *Pre-period*				
No. of 24 hour periods	18	18	18	18
	90 ±6	92 ±5	72 ±4	72 ±3
B. *Glycine*				
Preceding day	92	90	75	70
Injection periods (3 days)	91 (4·5)	85 (4·5)	65 (5)	70 (5)
	96 (3·5)	96 (3·5)	78 (5)	73 (5)
	91 (3·5)	92 (3·5)	70 (3)	69 (3)
C. *Alanine*				
Preceding day	90	93	72	80
Injection periods (3 days)	92 (4·5)	87 (4·5)	67 (4)	70 (4)
	80 (3·5)	93 (3·5)	72 (4)	75 (4)
	90 (3·5)	80 (3·5)	65 (3)	72 (3)
D. *Cystine*				
Preceding day	82	90	—	80
Feeding periods (2–3 days)	83 (5)	90 (3·5)	—	70 (3·5)
	80 (1)	90 (4)	—	69 (4)
	86 (4)	—	—	65 (3·5)

Table III.

Rabbit No. ...	III	IV	VII	VIII
Weight (kg.) ...	2·18	2·03	1·80	1·99
A. *Pre-period*				
No. of 24 hour periods	18 90±6	18 92±5	18 73±5	18 76±6
B. *Tyrosine*				
Preceding day	85	91	71	73
Feeding periods (2–3 days)	90 (5) 93 (5) 85 (3)	89 (4·5) 94 (10) —	76 (5) 70 (5) 73 (3)	70 (5) 75 (5) 80 (3)
Glutamic acid				
Preceding day	96	92	69	73
Feeding periods (2–3 days)	90 (5·5) 98 (3·0) 85 (4·0)	89 (7) 93 (5·5) 87	70 (5·5) 69 (3·0) 69 (4·0)	73 (7·0) 77 (5·5) 76

Glycocyamine

Rabbit No.	II		III	
	Preformed creatinine	Creatine	Preformed creatinine	Creatine
Preceding day	80	0	84	0
Feeding periods (2–3 days)	92	39 (3)	91	179 (3)
	95	47 (4)	68	269 (3)
	90	107 (3)	—	—
	94	161	—	—

Variable amounts of creatine (0–36 mg.) were found in the urine after injection of arginine and histidine, but similar amounts were excreted during the normal pre-periods and also after injection of Locke's solution. Consequently the occurrence of creatine after injection of the amino-acids cannot be regarded as significant.

Discussion.

The results in Table I show that the injection of arginine and histidine caused increases in urinary creatinine of the order of 10–40 %. The effects were definite in all the animals injected and although the reaction to arginine and histidine in any one animal was very similar, individual variations occurred. Single day injections into animal II gave increases of about 40 %, in I the increases were less (about 14 %). Injections on successive days caused these increases to persist; in some cases further rises occurred. These effects are in striking contrast to those obtained with other amino-acids. In Tables II and III it is seen that none of the amino-acids, glycine, alanine, cystine, tyrosine and glutamic acid, had any effect on the excretion of creatinine or creatine, whether administered subcutaneously or *per os*. The results in Tables II and III indicate further that the increases obtained with arginine and histidine could not have been due to specific dynamic action.

The large increases in creatine excretion as a result of feeding glycocyamine confirm the results of previous investigators. The preformed creatinine, except for a drop on the second day in animal III, remained unaffected.

It is highly significant that with arginine and histidine which have struc-

tural affinities to creatine and creatinine there are prompt and definite increases in creatinine, while with all the other amino-acids, which have no such direct relationship, there is no effect. Glycocyamine increases creatine excretion but has no effect on creatinine. An analysis of Table I indicates that arginine and histidine are identical in their effects upon urinary creatinine. From this it may be concluded that the iminazole nucleus in histidine and the guanidine nucleus in arginine undergo transformation into creatinine (either directly or through the intermediate stage of creatine).

On the other hand when the guanidine nucleus is introduced in the form of glycocyamine it is transformed directly into creatine which is excreted as such. This difference in effect may be due to the fact that arginine and histidine were injected and glycocyamine was administered *per os*, that is, that the locus of transformation in the former case was muscle and in the latter the liver. Reviewing the evidence in regard to the site of formation of creatinine from creatine Hunter [1928] comes to the conclusion that the liver plays no part in this change and that the muscles are most probably the chief site of creatinine formation. This does not exclude the possibility of creatine formation from glycocyamine in the liver; consequently the liver probably can convert glycocyamine into creatine but cannot convert creatine into creatinine. The creatine is formed from glycocyamine rapidly and in large amounts and is excreted as such. The rapidity with which creatinine arises from arginine and histidine suggests that its formation is a direct one, but the formation of creatine as an intermediary cannot be definitely excluded. The point raised in regard to the different effects obtained with parenteral or oral administration of creatine-creatinine precursors requires further detailed investigation and may lead to an explanation of the contradictory results encountered in the literature.

The above results although not conclusive indicate that arginine and histidine are normal precursors of creatine or creatinine and suggest that only those substances with close direct chemical relationships to creatine or creatinine can be taken into account in any attempt to elucidate the still rather obscure problem of the significant biochemical processes involved in creatine and creatinine metabolism and their final interrelationships.

Summary.

1. Injection of arginine and histidine into adult male rabbits gives rise to a 10–40 % increase in the elimination of urinary creatinine.

2. Feeding with glycocyamine has no effect on creatinine excretion but leads to a large output of creatine.

3. The amino-acids glycine, alanine (injected) and tyrosine, cystine, glutamic acid (ingested) have no effect on creatine or creatinine excretion.

4. It is suggested that the transformation of arginine and histidine into creatinine takes place in the muscles probably *via* creatine and that the glycocyamine-creatine change is a direct one taking place in the liver.

The expenses of this investigation have been defrayed by a grant from the Research Grant Board of the Union of South Africa, to which the authors express their thanks.

REFERENCES.

Abderhalden and Buadze (1929). *Arch. ges. exp. Med.* **65**, 1.
—— —— (1930). *Z. physiol. Chem.* **189**, 65.
Beard and Barnes (1931). *J. Biol. Chem.* **94**, 49.
Christman and Mosier (1929). *J. Biol. Chem.* **83**, 11.
Hunter (1928). Creatine and creatinine. (Longmans, London.)
Hyde and Rose (1929). *J. Biol. Chem.* **84**, 535.
Lewis, Dunn and Doisy (1918). *J. Biol. Chem.* **36**, 9.
Schrire and Zwarenstein (1932). *Biochem. J.* **26**, 118.
Zwarenstein (1928). *Biochem. J.* **20**, 307.

CCXXIV. THE INFLUENCE OF THE GONADS ON PROTEIN METABOLISM.

II. THE EFFECT OF INJECTIONS OF SALINE SUSPENSIONS OF TESTES AND TESTICULAR EXTRACTS ON URINARY CREATININE AFTER CASTRATION.

By ISIDORE SCHRIRE and HARRY ZWARENSTEIN.

From the Department of Physiology, University of Cape Town.

(*Received November 1st, 1932.*)

In a previous paper [Schrire and Zwarenstein, 1932] it was shown that castration of adult male rabbits led to a persistent increase in the excretion of urinary creatinine, that grafting of testes into castrated animals caused a decrease to the normal pre-castration level, and that after subsequent removal of the graft the usual castration effect on urinary creatinine was re-established. These results suggest the existence of an endocrine relationship between the testes and creatinine metabolism, but a definite conclusion can be arrived at only when evidence has been presented that the effects of castration can be compensated by injection of testicular suspensions and extracts. The results in regard to the effect of testicular injection on the increased elimination of creatinine after castration form the substance of this paper.

Experimental.

The procedure with regard to feeding, caging *etc.* was the same as reported in the previous communication. Three to four months after removal of the testes the usual castration effect on creatinine appeared. The testes of rabbits or portions of bull testis were finely divided with a sharp scalpel, the macerated liquid mass suspended in Locke's solution and injected subcutaneously by means of a large veterinary syringe and needle. The effects were controlled by injection of brain suspension prepared in the same way. The testicular extracts were prepared from the testes of bulls, pigs and rams according to the method described by Moore, McGee and co-workers [McGee, Juhn and Domm, 1928; Moore, Price and Gallagher, 1930]. The final extract was taken up in olive oil before injection.

Extracts of sheep's brain prepared by the same method or olive oil alone were injected as controls.

1 cc. of olive oil extract represents 94 g. of fresh testis or 57 g. of brain. In the first part of Table I (pre-period and 3 months castration figures) the

Table I. *Daily urinary creatinine.*

Rabbits Nos. 1, 2, 3. Normal animals.
„ 4 and 5. Castrated 14. iii. 32.
„ 6 and 7. Castrated 16. iii. 32.

Date	Normal (1)	Normal (2)	Normal (3)	Castrated (4)	Castrated (5)	Castrated (6)	Castrated (7)
			mg.				
A. Pre-periods and 3 months castration							
Jan. 17	95	63	63	94	60	74	86
Feb. 1	95	75	78	97	68	81	89
„ 29	97	75	81	94	72	79	93
Mar. 7	94	79	86	87	75	79	87
„ 29	91	—	—	89	—	79	74
Apr. 11	92	—	—	87	—	74	81
„ 25	93	—	—	91	—	79	86
May 16	93	—	—	90	—	93	92
June 15	97	—	—	94	—	84	96
B. Injection of saline suspensions of rabbit's testes							
July 11	79	92	81	116	104	107	100
„ 12	90	85	96	111	91	106	106
„ 13	95	85	79	120	106	114	106
„ 14	98	88	88	112	116*	125*	117
„ 15	92	92	86	103	75	93	108
„ 16	81	85	84	115	62	84	110
„ 17	96	85	82	112	110	105	113

C. Injection of saline suspensions of bull's testis and brain

	(1)	(3)	(5)	(6)
Sept. 9	92	78	116	98
„ 10	86	80	112	104
„ 11	83	74	108	111
„ 12	92	72	112	106
„ 13	92	80†	114†	108†
„ 14	90	92	120	116
„ 15	96	90	85	125
„ 16	100	79	105	121
„ 17	100	Died	124	127
„ 18	100	—	120	127

D. Injection of testicular and brain extracts and olive oil

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
July 23	78	70	85	120	105	105	109
„ 24	80	74	75	109	89	100	109
„ 25	76	80	90	109	104	109	120
„ 26	87	83	92	96	120	83	120
„ 27	93	75	80	96	106	75	109
„ 28	86	75	75	72	120	78	120
„ 29	86	70	51	86	104	100	114
„ 30	85	86	—	98	116	116	127

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
July 25	No injection	Brain 4 cc.	Oil 4 cc.	Testis 4 cc.	No injection	Testis 4 cc.	Brain 4 cc.
„ 26	„	„	„	Testis 6 cc.	„	Testis 6 cc.	„
„ 27	„	„	„	Testis 10 cc.	„	Testis 10 cc.	„
„ 28	„	Oil 4 cc.	Testis 10 cc.	Testis 10 cc.	„	Testis 10 cc.	Oil 4 cc.

* On July 14 Nos. 5 and 6 injected with saline suspension of testis.
† On Sept. 13 No. 5 injected with saline suspension of testis. Nos. 3 and 6 injected with saline suspension of brain.

dates refer to the first of five 24-hour periods in each case. The mean of the daily creatinine output in mg. is given for each series of 24-hour periods. Rabbits Nos. 2, 3 and 5 were allowed to run free for about 4 months when they were returned to the metabolism cages.

Discussion.

The post-castration rise in urinary creatinine commencing 3 to 4 months after removal of the testes confirms the results reported previously. Examination of the data shows further that injection of saline suspensions of rabbit's or bull's testis led after a slight rise to a fall in creatinine to the pre-castration level. On the other hand, injections of brain suspension had no such effect on either the normal or castrated animal. With rabbits' testes the effect persisted for two days, after which the creatinine rose to the castration level. With bull's testis the fall occurred on the second day after injection after which there was again a rapid increase.

Injection of testicular extracts also caused a decrease to the normal pre-castration level. In rabbit No. 4 the final injection on July 28th led to a creatinine output for that day which was below normal. The decrease persisted throughout the injection periods, rising again when injections ceased. Injections of brain extract or of olive oil had no effect. Rabbit No. 3, a normal animal, was injected on July 28th with testicular extract instead of with olive oil and this led to an immediate drop in creatinine excretion.

The decrease in the high creatinine of castration caused by injections of testicular suspensions or extracts and the subsequent post-injection rise are effects which are in complete agreement with those obtained with grafting of testes and subsequent removal of the graft.

The results as a whole thus afford strong evidence in favour of the existence of an endocrine relationship between the testes and creatinine metabolism. This relationship may be a direct one or it may be an indirect one through some other organ of internal secretion. The fact that the castration effect only appears after a latent period of several months suggests that it is the result of a slow, gradual change in another endocrine organ. This hypothesis is supported by the results of recent work on the relation between the gonads and the pituitary and the pituitary and creatinine metabolism. Martins and Rocha [1931] review the evidence in regard to the regulation of pituitary activity by the testes and report their results on parabiotic experiments in rats. They come to the conclusion that as a result of castration a pituitary hypertrophy, associated with the presence of "castration cells" in the anterior lobe, occurs and the organism becomes flooded with anterior pituitary hormones. The gonads normally exert an inhibitory effect on the pituitary gland.

Braier [1931] has shown that hypophysectomised dogs on a complete diet eliminate 24 % less creatinine than the controls. This difference is accentuated

in fasting animals, removal of the pituitary leading to a 35–40 % decrease in creatinine excretion. This effect was obtained as early as 30 days after operation. Evidence in regard to the effect of injections of pituitary extracts on urinary creatinine is lacking but it is reasonable to assume on the basis of these results that increased activity of the pituitary gland associated with hypertrophy of the anterior lobe occurring as the result of castration would lead to an increased elimination of creatinine.

On the assumption that castration causes marked hypertrophy of the anterior lobe of the pituitary in male rabbits the castration effect on creatinine excretion is explicable as being due to the unantagonised hyperactivity of the anterior lobe.

The continuous stimulating effect of the pituitary is rapidly and effectively antagonised by injection of saline suspensions of testes or testicular extracts and more slowly but as effectively by grafting testes into castrated animals. In both cases the creatinine excretion drops to normal. The effect is a transient one with injection but persists as a result of grafting. Removal of the graft almost immediately brings into operation the effect of pituitary hyperactivity and the creatinine rises to the typical castration level; the same effect is seen during the post-injection periods.

SUMMARY.

1. Injection of saline suspensions of testes or of testicular extracts causes the high urinary creatinine of castration to decrease to the normal pre-castration level.

2. It is pointed out that the results reported above together with those in a previous paper afford strong evidence in favour of the existence of an endocrine relationship between the testes and creatinine metabolism.

3. It is suggested that the castration effect is an indirect one through the anterior lobe of the pituitary gland.

We have to thank Dr L. P. Bosman and Mr D. G. Duncan for assistance in the preparation of extracts.

REFERENCES.

Braier (1931). *Rev. Soc. Arg. Biol.* **7**, 340.
Martins and Rocha (1931). *Endocrinology*, **15**, 421.
McGee, Juhn and Domm (1928). *Amer. J. Physiol.* **87**, 406.
Moore, Price and Gallagher (1930). *Amer. J. Anat.* **45**, 71.
Schrire and Zwarenstein (1932). *Biochem. J.* **26**, 118.

CCXXV. SOME NEW ANTISEPTICS RELATED TO INDAN-1:3-DIONE.

By FRANK ARNOLD ROBINSON, ARTHUR JAMES SUTHERS and THOMAS KENNEDY WALKER.

From the College of Technology, University of Manchester.

(*Received September 29th, 1932.*)

Many compounds containing the 1 : 3-diketo-system, or the enolic form of the same, have been found to possess definite forms of physiological activity, and such properties may be particularly evident in molecules in which the 1 : 3-diketo-grouping constitutes part of a ring structure to which one or more alkyl or acyl groups are attached in certain positions. Cases in point are furnished by the hypnotic derivatives of barbituric acid, in which a *gem*-dialkyl group is situated between the two carbonyls; further instances are those of filicic acid and aspidinol, nuclear units of complex and more active substances present among the constituents of *Aspidium filix-mas* and of other ferns [*cf.* Henry, 1923].

The nucleus of filicic acid contains the grouping

$$\overset{1}{\text{—CO—}}\overset{2}{\text{C}} : (\text{CH}_3)_2\text{—}\overset{3}{\text{CO}}\text{—}\overset{4}{\underset{|}{\text{C}}}=$$

and possesses a substituent acyl group attached to carbon atom number 4. Recognition of the presence of such a system in the naturally occurring anthelmintics has led to the examination of the properties of synthetic acyl derivatives of resorcinol and of phloroglucinol [Karrer and Rosenfeld, 1921] and these have been found to possess different degrees of anthelmintic activity.

Alkyl derivatives of resorcinol and of phloroglucinol, particularly a hexyl derivative of the former [Johnson and Lane, 1921; Leonard, 1924; Dohme, Cox and Miller, 1926; Klarmann, 1926; Klarmann and Figdor, 1926] possess marked antiseptic potency and it is noteworthy that lupulon, a highly antiseptic phloroglucinol derivative occurring in the soft resin of hops [Walker, 1924; 1925; Wöllmer, 1925; Wieland, Marz and Hoek, 1925; Wieland and Marz, 1926; Pyman, 1927], bears a very close structural relationship to filicic acid, an additional indication that antiseptic activity of a certain kind and anthelmintic activity are properties connected with the particular type of molecular architecture possessed by these substances.

Consideration of these and of similar relationships led one of us to make further investigation of the extent to which the 1 : 3-diketo-system, in conjunction with other groups, can condition or enhance physiological activity,

particularly antiseptic potency, and recently it was demonstrated [Black, Shaw and Walker, 1931; Walker *et al.*, 1931] that a number of derivatives of indan-1 : 3-dione, produced by attachment of the malonyl or mono-*n*-alkylmalonyl group to the nuclei of phenols, exert considerable bacteriostatic effects upon certain micro-organisms. In three cases it was shown that, starting from monoethylmalonyl chloride, the coupling of an homologous series of mono-*n*-alkylmalonyl chlorides with a given phenol resulted in the production of an homologous series of 2-*n*-alkylindan-1 : 3-diones of progressively increasing antiseptic power; and in the series derived from *p*-cresol such increase in power attained its limit with the compound containing the *n*-heptyl radical.

In view of this behaviour and of the fact that similar progressive increase of antiseptic potency has been noted in the homologous series of *n*-alkylresorcinols, where it culminates in the *n*-hexyl compound [Dohme, Cox and Miller, 1926; Klarmann, 1926], it appeared probable that condensations of *n*-amyl- and *n*-hexylmalonyl chlorides with suitable *n*-amyl, *n*-hexyl and *n*-heptyl derivatives of phenol would give rise to indan-1 : 3-diones of considerably greater antiseptic potency than those hitherto prepared.

Accordingly, a series of *p*-*n*-alkylphenols was synthesised and coupled with various *n*-alkylmalonyl chlorides, and investigation of the properties of the products has fully justified expectations as to their degrees of antiseptic activity. It was also intended originally to apply this reaction for the production of indan-1 : 3-diones to *C*-alkyl derivatives of *o*-, *m*-, and *p*-cresol but, whilst experiments with 3-*n*-amyl-*p*-cresol and indan-1 : 3-diones derived from it were in progress, Coulthard, Marshall and Pyman [1930] published an account of the syntheses and germicidal activities of several homologous series of phenols, including *C*-alkyl derivatives of *o*-, *m*- and *p*-cresol, and the data given in their paper have rendered unnecessary further work on the lines we had in view.

The constitutions of the new indan-1 : 3-diones and the numbers assigned to them for reference purposes are shown in Tables II and III, compounds I to XIV, inclusive, possessing structure (A), and compounds XV to XVIII, inclusive, possessing structure (B). Compound (I) was prepared during the course of a previous investigation [Walker *et al.*, 1931] and is included here in order to complete series I to VII.

The new substances exercise selective antiseptic action, for whilst they inhibit, even in very low concentrations, the proliferation of many Gram-positive bacilli and cocci, they exert little or no effect on the reproduction of certain Gram-negative organisms, relatively large doses of the substances failing to restrain the development of *Bact. coli commune*, *Bact. pyocyaneum* and *Bact. prodigiosum*, in broth or other media.

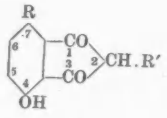
Table I permits comparison of the bacteriostatic powers of the members of the series of *p*-*n*-alkylphenols and of that of 3-*n*-amyl-*p*-cresol, whilst comparisons of the powers of the new indan-1 : 3-diones prepared from these substances may be made from the data in Tables II and III. The inhibiting

Table I.

Substance	Bacteriostatic concentration expressed as 1 g. of substance in x cc. of medium	Equimolecular phenol coefficient of bacteriostatic power	Equimolecular phenol coefficient of bactericidal power (calculated from phenol coefficients obtained by use of Rideal-Walker method)
Phenol	1 in 660	—	—
p-Cresol	1 in 1,580	2·7	2·87
p-Ethylphenol	1 in 1,660	3·2	9·7
p-*n*-Propylphenol	1 in 8,300	16·2	28·9
p-*n*-Butylphenol	1 in 25,000	59·8	111·7
p-*n*-Amylphenol	1 in 76,900	189	181·4
p-*n*-Hexylphenol	1 in 125,000	355	170·4
p-*n*-Octylphenol	1 in 550,000	1827	—
3-*n*-Amyl-*p*-cresol	1 in 71,400	205	473

[The concentrations of *p*-*n*-amylphenol and *p*-*n*-hexylphenol necessary to suppress the proliferation of *Bact. coli commune* in broth (p_H 6·1) for 48 hours at 37° were found to be 1 in 8300 and 1 in 12,500, respectively, whilst under the same conditions and in the same space of time either compound at a concentration of 1 in 5000 was found to kill this organism. A concentration of 1 in 100,000 of *p*-*n*-octylphenol killed *Bacterium C* in 48 hours but *Bact. coli commune* was not killed in 48 hours by this compound at a concentration of 1 in 25,000 (saturated solution).]

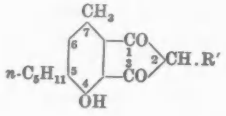
Table II.



(A)

		Bacteriostatic concentration, expressed as 1 g. of substance in x cc. of medium	Equimolecular phenol coefficient of bacteriostatic power
(I)	R=CH_3	1 in 45,000 {1 in 9,080 at p_H 6·7; 1 in 3,630 at p_H 7·7}	170
(II)	R=C_2H_5	1 in 125,000	490
(III)	R=n-C_3H_7	1 in 222,000	920
(IV)	R=n-C_4H_9	1 in 555,000	2,430
(V)	R=n-C_5H_{11}	1 in 1,250,000	5,740
(VI)	R=n-C_6H_{13}	1 in 3,333,000	16,060
(VII)	R=n-C_8H_{17}	1 in 5,000,000	26,330
(VIII)	R=n-C_4H_9	1 in 50,000 {1 in 20,000 at p_H 6·7; 1 in 6,660 at p_H 7·7}	196
(IX)	R=n-C_6H_{13}	1 in 500,000	2,187
(X)	R=n-C_5H_{11}	1 in 3,333,000	16,060
(XI)	R=n-C_6H_{13}	1 in 5,000,000 {1 in 2,500,000 at p_H 6·7; 1 in 500,000 at p_H 7·7}	25,212
(XII)	R=C_2H_5	1 in 250,000 {1 in 125,000 at p_H 6·7; 1 in 62,500 at p_H 7·7}	1,093
(XIII)	R=n-C_5H_{11}	1 in 1,000,000	5,044
(XIV)	R=n-C_6H_{13}	1 in 5,000,000 {1 in 1,250,000 at p_H 6·7; 1 in 833,300 at p_H 7·7}	26,330

Table III.



(B)

		Bacteriostatic concentration, expressed as 1 g. of substance in x cc. of medium	Equimolecular phenol coefficient of bacteriostatic power
(XV)	R'=n-C_3H_7	1 in 833,300	3,830
(XVI)	R'=n-C_4H_9	1 in 1,666,600	8,030
(XVII)	R'=n-C_5H_{11}	1 in 3,333,000	16,800
(XVIII)	R'=n-C_6H_{13}	1 in 250,000	1,330

concentrations were determined by the turbidity method in lemco-peptone broth (p_H 6·1, unless otherwise stated), the organism employed being *Bacterium C* (National Collection of Type Cultures, Lister Institute). Incubation in the presence of different doses of each antiseptic was allowed to proceed for 48 hours at 37° and the results of all the tests were confirmed by triplicate determinations. *Bacterium C* was selected originally for the purpose after preliminary trials had shown that its behaviour towards this class of antiseptics is representative of that of a number of Gram-positive bacilli and cocci; moreover *Bacterium C* develops rapidly, it may be cultured over considerable periods of time without change of properties, and, in other ways, it is very suitable for use in determinations of bacteriostatic power.

In Tables I, II and III, the third column in each case shows the bacteriostatic powers of the several compounds compared with that of phenol and expressed on an equimolecular basis[1], *i.e.* equimolecular phenol coefficient of bacteriostatic power = (phenol coefficient of bacteriostatic power × molecular weight of substance) ÷ molecular weight of phenol. The figures in the fourth column of Table I have been calculated from phenol coefficients given by Coulthard, Marshall and Pyman [1930], and they are shown here for purposes of comparison. It is of interest to note in the case of the *p-n*-alkylphenols that while both the bacteriostatic activity towards *Bacterium C* and the bactericidal activity towards *Bact. typhosum* increase with the number of carbon atoms in the side-chain, the bactericidal activity attains its maximum in the *p-n*-amyl derivative whereas the bacteriostatic activity continues to increase along the series beyond the *p-n*-amyl derivative and may not have attained its maximum in the *p-n*-octyl derivative, the highest member examined. The *p-n*-heptyl derivative of phenol was not prepared since oenanthic acid was not available at the time this work was undertaken.

Reference has already been made to the fact that in the homologous series of 4-hydroxy-7-methyl-2-*n*-alkylindan-1 : 3-diones antiseptic activity was found to increase progressively until it attained a maximum intensity in the 2-*n*-heptyl derivative. Similar behaviour is to be noted in two homologous series of this type shown in Tables II and III, respectively. In Table II, compounds IX, VI, XI and XIV are members of such an homologous series and it would appear that the antiseptic activity has culminated in XIV, where R' = *n*-hexyl, since the equimolecular phenol coefficient of this compound is only very slightly greater than that of XI. The four compounds in Table III show very definitely the same kind of behaviour; here the 2-*n*-amyl derivative possesses the greatest antiseptic power, the 2-*n*-hexyl compound proving to be relatively weak.

Scrutiny of Table II also shows that progressive increase of antiseptic potency occurs in the series of compounds prepared by coupling a given alkylmalonyl chloride with an homologous series of *p-n*-alkylphenols, and if

[1] Morgan, Cooper and Burtt [1923] have pointed out that it is preferable to compare phenol coefficients on an equimolecular basis.

we consider the series I to VII, inclusive, it is seen that bacteriostatic potency is very considerably higher in the case of the 7-*n*-octyl derivative VII than in that of the 7-*n*-hexyl derivative VI. Since the 7-*n*-heptyl and the 7-*n*-nonyl derivatives were not prepared, it is not possible to decide from the present data whether the 7-*n*-octyl derivative is the most potent compound of this type which could be synthesised. Further synthetical work in this series was not practicable, however, for great difficulty was encountered in separating pure substances from the highly resinous reaction products which were obtained when components containing these larger alkyl groups were employed in the condensation.

Bacteriostatic and bactericidal action of indan-1:3-diones on a variety of micro-organisms.

In order to obtain information on broader lines concerning the inhibitory and lethal effects of the new indan-1 : 3-diones, three of these compounds (*viz.* X, XIV and XVII) which, in tests against *Bacterium C*, had exhibited considerable bacteriostatic powers, were employed in experiments with the following organisms: *Bact. pyocyaneum* Flügge (Fildes III), *Bact. prodigiosum* Cohn, *Bact. coli* Escherich, *Staphylococcus albus* Rosenbach, *Bac. megatherium* de Bary, *Bac. subtilis* (Ehrenberg) Cohn, *Bac. mycoides* Flügge and two strains of *Mycobact. phlei* (Möller) Lehmann and Neumann. These organisms, with the exception of the specimen of *S. albus*, were obtained from the National Collection of Type Cultures, Lister Institute.

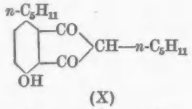
The determinations of bacteriostatic and bactericidal activity were made by the turbidity method previously employed in the case of *Bacterium C*, living organisms being sought for in subcultures when visual inspection of the tubes failed to detect growth. The results are shown in Table IV where, for purposes of comparison, the effects of these substances on *Bacterium C* are also reproduced.

These determinations have served to emphasise the highly selective action of the substances, since *Bact. pyocyaneum*, *Bact. prodigiosum* and *Bact. coli* were not prevented from multiplying by concentrations many times greater than those found necessary to kill the organisms cited in Table IV. Marked selectivity is also seen in the cases of *S. albus* and *M. phlei*, compound X exerting intense bacteriostatic effects upon the former organism, while the latter organism appears to be particularly susceptible to the influence of compound XIV.

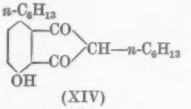
It was not considered necessary to determine the exact bactericidal concentration in more than half of the cases studied; in other instances it was ascertained whether the lethal dose lay above or below a given figure. The data shown in Table IV are adequate, however, to indicate that in some cases (*e.g.* that of *M. phlei*) the dose of antiseptic which is just sufficient to inhibit growth over a period of 48 hours is also lethal in that time, that is to say, in such cases the germicidal concentration is only slightly higher than the highest

Table IV. *Results illustrating the bacteriostatic and bactericidal activities of indan-1:3-dione derivatives towards various micro-organisms.*

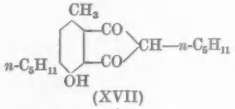
(*a*) = bacteriostatic concentration in broth (p_H 6·1) at 37° for 48 hours; (*b*) = bactericidal concentration in broth (p_H 6·1) at 37° for 24 hours.



(X)



(XIV)



(XVII)

Organism	(X) (*a*)	(X) (*b*)	(XIV) (*a*)	(XIV) (*b*)	(XVII) (*a*)	(XVII) (*b*)
**Bacterium C*	1 : 3,333,000	>1 : 166,000	1 : 5,000,000	<1 : 166,000	1 : 3,333,000	<1 : 166,000
Bac. megatherium	1 : 2,500,000	>1 : 500,000	—	—	1 : 2,500,000	>1 : 1,666,000
Bac. subtilis	1 : 2,500,000	<1 : 500,000	1 : 5,000,000 (1 : 1,250,000 at p_H 7·7)	1 : 1,430,000 (1 : 833,000 at p_H 7·7)	1 : 2,500,000	>1 : 1,000,000
Bac. mycoides	1 : 2,500,000	<1 : 500,000	1 : 5,000,000 (1 : 1,250,000 at p_H 7·7)	1 : 2,000,000 (1 : 1,250,000 at p_H 7·7)	1 : 3,333,000	1 : 1,666,000
S. albus	<1 : 10,000,000	<1 : 500,000	1 : 3,333,000 (1 : 1,000,000 at p_H 7·7)	1 : 1,666,000 (1 : 833,000 at p_H 7·7)	1 : 2,500,000	1 : 1,666,000
M. phlei	(i)	(i)	(i)	(i)	(i)	(i)
(i) strain 525	1 : 5,000,000	<1 : 500,000	1 : 11,110,000	1 : 11,110,000	1 : 3,333,000	1 : 3,333,000
(ii) strain 54			(ii) 1 : 20,000,000 (ii) (1 : 1,428,000 at p_H 7·7)	(ii) 1 : 20,000,000 (ii) (1 : 1,428,000 at p_H > 7·7)	(ii) 1 : 3,333,000	(ii) 1 : 2,000,000

* This unclassified organism was designated provisionally as *Bacterium C* by Chapman but, since it gives rise to spores it could be designated, according to the convention of Lehmann and Neumann, as *Bacillus C*.

concentration of antiseptic in the presence of which growth can occur with comparative freedom. In other cases, according to the particular organism, the concentration of antiseptic necessary to produce germicidal effects within 24 hours may be as much as 20 times higher than that which is sufficient to inhibit growth during the same period (for example, *Bacterium C* and compound X).

In view of these results it was thought of interest to obtain further information as to the intensity of the bactericidal activity of the substances towards Gram-positive organisms and, accordingly, the action of compound XI on *Bac. megatherium*, *Bac. subtilis*, *Bac. mycoides* and *S. albus* was studied under the conditions of the Rideal-Walker method. The substance (0·1 g.) was first dissolved in 100 cc. of 0·02 *N* Na_2CO_3 and, by dilution of aliquot portions of this stock solution, with sterile water, the concentrations required were obtained (Table V).

The bactericidal action of these indan-1 : 3-diones is seen, therefore, to be rapid at higher concentrations, and still reasonably rapid even at low concentrations, and, generally, in their antiseptic effects the substances are strikingly similar to the triphenylmethane dyes of the type of crystal violet which, as Churchman [1926] has shown, are much more powerfully bacteriostatic towards Gram-positive organisms such as staphylococcus, diphtheroids and

Table V.

Organism	Time, in minutes, necessary for completion of the lethal action of compound XI at concentrations of 1 : 10,000	1 : 100,000	1 : 1,000,000
Bac. megatherium	<10	—	<30
Bac. subtilis	<10	<30	—
Bac. mycoides	<10	<60	—
S. albus	<10	—	<60

Bact. coli was killed in 30 minutes but not in 15 minutes by compound XI at a concentration of 1 : 1000.

aerobic spore-forming bacilli than towards Gram-negative organisms like *Bact. coli*. With the indan-1 : 3-diones, however, this selectivity of action is decidedly more pronounced than with the triphenylmethane dyes, and this fact would appear to indicate that the former compounds could be utilised very successfully in the separation of coliform organisms from certain mixed cultures.

The effect of hydrogen ion concentration on the antiseptic activity of indan-1:3-diones.

Tables II and IV contain sufficient data concerning the effects of hydrogen ion concentration to indicate clearly that the antiseptic activities of the substances examined are greatly diminished by adjustment of the medium from the acid range of the scale towards the neutral point, and that this effect becomes still more marked when the medium is rendered faintly alkaline. It is also evident that no general statement can be made regarding the relative effects likely to be produced by alterations in the reaction of the medium, since two compounds which exercise approximately equal inhibitory actions upon a given organism at one particular hydrogen ion concentration, may manifest widely divergent activities towards the same organism at other hydrogen ion concentrations.

The effect of serum on the antiseptic activity of indan-1:3-diones.

It was considered desirable to ascertain the effect of serum upon the bactericidal activities of indan-1 : 3-dione derivatives, and Prof. C. H. Browning very kindly undertook to examine one of the new compounds from this point of view. The substance chosen was 4-*hydroxy*-7-n-*hexyl*-2-n-*amyl indan*-1 : 3-*dione* (compound XI) which, as previous tests had shown, is one of the most potent of the new compounds. He reported as follows:

"0·05 g. was dissolved in 5 cc. alcohol and 5 cc. water added; the precipitate which formed was dissolved by the addition of a drop of Na_2CO_3 solution. This 0·5 % stock solution was stable and from it further dilutions in water were made. A volume not exceeding 0·1 cc. of various dilutions was then added to a series of tubes each containing 1 cc. 0·7 % peptone water medium or ox-serum (previously heated at 56°), so as to yield a wide range of concentrations of the antiseptic. In peptone water precipitation occurred

with 1 : 20,000 of the substance, but 1 : 2000 in serum gave no precipitate. The tubes were then inoculated with 0·1 cc. of a 1 : 1000 dilution in saline of a 24 hours' fluid culture of *S. aureus* or *Bact. coli*. The results were read after 48 hours' incubation at 37°, both naked-eye evidence of growth and the presence of living organisms, as tested by subculture, being determined.

The *Staphylococcus* in peptone water was sterilised by 1 : 400,000, but in serum 1 : 2000 failed to sterilise. In the case of *Bact. coli*, 1 : 2000 failed to sterilise in peptone water and in serum."

Syntheses of acylphenols and reduction of these to alkylphenols.

The *p*-*n*-alkyl derivatives of phenol examined by Coulthard, Marshall and Pyman [1930] were obtained by reduction of the corresponding *p*-acylphenols by Clemmensen's method. These authors prepared *p*-propionyl, *p*-*n*-valeryl and *p*-*n*-hexoylphenols by heating phenol with the appropriate fatty acid and anhydrous zinc chloride, a process due originally to Nencki. We found that much better yields of these three ketones were obtained by employing the Fries isomerisation, a method used by Coulthard, Marshall and Pyman [1930] to obtain the corresponding derivatives of the three cresols. In our experiments the acid chloride (1 mol.) was added to phenol (1 mol.) and 30 minutes later the mixture was warmed on a water-bath for 60 minutes. The resulting ester of the phenol was then diluted with three times its volume of freshly distilled nitrobenzene, and aluminium chloride (1·1 mols.) in a finely powdered condition was added in small quantities at a time. The reaction was completed by heating on a water-bath for 60 minutes. The material was then submitted to steam-distillation in order to remove nitrobenzene and to separate the volatile *o*-hydroxyarylketone from the non-volatile *p*-isomeride. The latter was isolated by extraction with ether and distillation under low pressure. The yields were: *p*-propionylphenol, 25 %; *p*-*n*-butyrylphenol, 18 % (Coulthard, Marshall and Pyman obtained 19 % in absence of nitrobenzene); *p*-*n*-valerylphenol, 37 %; *p*-*n*-hexoylphenol, 58 %.

The preparation of 3-*n*-valeryl-*p*-cresol (65 % yield) was carried out under the conditions used by Coulthard, Marshall and Pyman, *i.e.* the isomerisation was conducted at 160° in absence of nitrobenzene.

p-Acetylphenol was prepared according to a method described by Nencki [1897], which consisted in treating a mixture of phenol and acetyl chloride in carbon disulphide solution with ferric chloride. The yield was 60 %. The reduction of the above-mentioned *p*-hydroxyarylketones to the corresponding alkylphenols by Clemmensen's method was effected in every case with a yield of 80 % or over.

p-*n*-Octylphenol has been described recently by Sandulesco and Girard [1930]. We prepared it as follows. *n*-Caprylic acid (73 g.) on treatment with thionyl chloride (88 g.) gave 57 g. (70 % yield) of the acid chloride, which boiled at 101–102°/18 mm. This was added to freshly distilled phenol (33 g.) and, after the resulting reaction had moderated, the mixture was warmed for

60 minutes on a steam-bath and subsequently diluted with 200 cc. of freshly distilled nitrobenzene. Finely powdered anhydrous aluminium chloride (52 g.) was now added in small portions, and the reaction was completed by heating for an hour at 75°. The reaction product was poured on ice, treated with hydrochloric acid and submitted to steam-distillation. The non-volatile organic residue was taken up in ether and was subsequently fractionated under reduced pressure. Some material which distilled up to 180° at 13 mm. probably contained a little *o*-*n*-caprylylphenol; the *p*-*n*-caprylylphenol subsequently passed over at 215 to 220°/15 mm. and solidified on standing. This crude fraction (30 g., *i.e.* 40 % yield) was not further purified but was reduced by heating with 100 g. of amalgamated zinc-wool and 600 cc. of dilute hydrochloric acid (1 vol. concentrated acid : 2 vols. water) on a water-bath at 100° during 16 hours, the acid being added in portions from time to time. (The zinc-wool had been amalgamated by treatment for 5 minutes with a 5 % aqueous solution of mercuric chloride.) The reduction product, isolated by extraction with ether, was distilled under reduced pressure and the fraction boiling at 195–205°/15 mm. (Sandulesco and Girard give 169°/10 mm.) was collected. This crystallised in colourless needles, on standing, and the yield was 55 %. Owing to the fact that the melting-point was low and indefinite (*ca.* 41°) the substance was characterised as the p-*nitrobenzoate*, which was prepared by heating 3 g. of the phenol with 3 g. of *p*-nitrobenzoyl chloride in 20 cc. of pyridine on a water-bath for 2 hours. The reaction mixture was poured into dilute hydrochloric acid and the solid product was separated, dried and crystallised from acetone. It melted at 81·5°. (Found, by micro-method: N, 4·07 %, $C_{21}H_{25}O_4N$ requires N, 3·94 %.)

A communication by Sandulesco and Girard [1930], which was published almost simultaneously with that of Coulthard, Marshall and Pyman, also contains an account of the syntheses of *o*- and *p*-acyl derivatives of phenol and of their subsequent reduction to the corresponding alkyl derivatives, the methods employed being very similar to those used independently by us and by Coulthard, Marshall and Pyman. Sandulesco and Girard state that somewhat higher yields of the *p*-acyl derivatives of phenol are obtained when working in the absence of nitrobenzene. Their yields of *p*-alkyl derivatives of phenol obtained by use of Clemmensen's reduction method are of the same order as those obtained by us.

Syntheses of 4-hydroxyindan-1:3-diones.

A general method, and modifications of the same, for the preparation of substances of this class were described in a former communication [Walker *et al.*, 1931]. The 17 new compounds examined in the course of the present investigation were all prepared by this same general process, namely, condensation of an alkylmalonyl chloride with an alkylphenol in nitrobenzene solution in the presence of anhydrous aluminium chloride. In some cases, however, difficulties were encountered in purifying the product, and this was

the case notably in those instances where R and R' were alkyl groups containing more than four carbon atoms. Increase in length of the alkyl chain in the malonyl chloride component was accompanied by an increasing tendency to resin formation, particularly if the reaction in nitrobenzene solution was conducted above 80° and, on the other hand, the employment of a temperature lower than 70° resulted in a low yield since a smaller percentage of the components interacted. In this latter event the product was contaminated with relatively large amounts of alkylmalonic acid and unchanged alkylphenol, and the latter, owing to low volatility, could not be removed in steam. In such cases the following procedure was adopted for the isolation and purification of the indan-1 : 3-dione. The nitrobenzene solution of the reaction products was poured on to ice and treated with sufficient hydrochloric acid to take the aluminium into solution. The nitrobenzene was removed in steam and the cooled residue was extracted thoroughly with ether. The ethereal extract was then washed many times with water to remove all traces of alkylmalonic acid, after which it was extracted very thoroughly with successive portions of a large bulk of a 7·5 % aqueous solution of sodium carbonate, followed by an extraction with cold water. This removed the indan-1 : 3-dione in the form of its sodium salt, and unchanged alkylphenol was left in the ether. The combined sodium carbonate extracts together with the final aqueous extract were acidified with hydrochloric acid and the free indan-1 : 3-dione was taken up in ether when, after drying and removal of the ether, it solidified and was recrystallised from appropriate solvents.

All the new indan-1 : 3-diones are colourless solids which are practically insoluble in cold water and soluble in aqueous solutions of alkali metal hydroxides or alkali metal carbonates. They are generally soluble in, and crystallise well from, the usual organic solvents, the most suitable of which for purposes of purification were found to be acetone and methyl alcohol in the hot, and an acetone-light petroleum mixture.

The analytical data and melting-points are given in Table VI. The combustions were performed by the micro-method.

Table VI.

Substance (see Tables II and III)		Formula	M.P.	Found		Calculated	
				% C	% H	% C	% H
4-Hydroxy-7-ethyl-2-*n*-butylindan-1:3-dione	(II)	$C_{15}H_{18}O_3$	111°	73·0	7·5	73·2	7·3
4-Hydroxy-7-*n*-propyl-2-*n*-butylindan-1:3-dione	(III)	$C_{16}H_{20}O_3$	122–123	74·5	8·1	73·8	7·7
4-Hydroxy-2:7-di-*n*-butylindan-1:3-dione	(IV)	$C_{17}H_{22}O_3$	120	74·9	7·9	74·4	8·0
4-Hydroxy-7-*n*-amyl-2-*n*-butylindan-1:3-dione	(V)	$C_{18}H_{24}O_3$	110	75·2	8·4	75·0	8·3
4-Hydroxy-7-*n*-hexyl-2-*n*-butylindan-1:3-dione	(VI)	$C_{19}H_{26}O_3$	118–119	76·0	8·7	75·5	8·6
4-Hydroxy-7-*n*-octyl-2-*n*-butylindan-1:3-dione	(VII)	$C_{21}H_{30}O_3$	105	76·4	9·35	76·4	9·1
4-Hydroxy-7-*n*-butyl-2-ethylindan-1:3-dione	(VIII)	$C_{15}H_{18}O_3$	129	73·2	7·3	73·2	7·3
4-Hydroxy-7-*n*-hexyl-2-ethylindan-1:3-dione	(IX)	$C_{17}H_{22}O_3$	120	74·1	8·4	74·4	8·0
4-Hydroxy-2:7-di-*n*-amylindan-1:3-dione	(X)	$C_{19}H_{26}O_3$	114–115	75·2	9·0	75·5	8·6
4-Hydroxy-7-*n*-hexyl-2-*n*-amylindan-1:3-dione	(XI)	$C_{20}H_{28}O_3$	100	75·8	8·9	75·9	8·8
4-Hydroxy-7-ethyl-2-*n*-hexylindan-1:3-dione	(XII)	$C_{17}H_{22}O_3$	110	74·6	7·9	74·4	8·0
4-Hydroxy-7-*n*-amyl-2-*n*-hexylindan-1:3-dione	(XIII)	$C_{20}H_{28}O_3$	98–99	75·5	9·0	75·9	8·8
4-Hydroxy-2:7-di-*n*-hexylindan-1:3-dione	(XIV)	$C_{21}H_{30}O_3$	95–96	76·0	9·3	76·4	9·1
4-Hydroxy-5-*n*-amyl-7-methyl-2-*n*-propylindan-1:3-dione	(XV)	$C_{18}H_{24}O_3$	149	75·5	8·0	75·0	8·3
4-Hydroxy-5-*n*-amyl-7-methyl-2-*n*-butylindan-1:3-dione	(XVI)	$C_{19}H_{26}O_3$	119–120	76·1	8·9	75·5	8·6
4-Hydroxy-7-methyl-2:5-di-*n*-amylindan-1:3-dione	(XVII)	$C_{20}H_{28}O_3$	90–92	76·1	8·6	75·9	8·8
4-Hydroxy-5-*n*-amyl-7-methyl-2-*n*-hexylindan-1:3-dione	(XVIII)	$C_{21}H_{30}O_3$	143–144	76·3	9·1	76·4	9·1

SUMMARY.

1. Seventeen new alkyl derivatives of 4-hydroxyindan-1 : 3-dione, all of which possess antiseptic power, have been prepared by the condensation of mono-*n*-alkylmalonyl chlorides with *p*-*n*-alkyl derivatives of phenol and with 3-*n*-amyl-*p*-cresol, in the presence of aluminium chloride.

2. Examination of these products shows that when a given mono-*n*-alkylmalonyl chloride is condensed with the lower members of the homologous series of the *p*-*n*-alkyl derivatives of phenol, the antiseptic powers of the members of the resulting homologous series of 4-hydroxyindan-1 : 3-diones increase progressively as the series is ascended. The limits of such increases have not been determined in the present investigation.

3. Two sets of results now obtained confirm a former observation that the condensation of several consecutive lower members of the homologous series of mono-*n*-alkylmalonyl chlorides with an appropriate phenol results in the formation of an homologous series of hydroxyindan-1 : 3-diones, the members of which exhibit antiseptic power which increases progressively to a maximum value as the series is ascended. Such maximum value appears usually to be attained in the 2-*n*-amyl-, 2-*n*-hexyl-, or 2-*n*-heptyl-compound.

4. The antiseptic activity of these 4-hydroxyindan-1 : 3-diones is highly selective in character. In very low concentrations they inhibit the proliferation of Gram-positive bacilli and cocci, as shown by tests against *Bac. megatherium*, *Bac. subtilis*, *Bac. mycoides*, *S. albus* and *S. aureus*. At higher concentrations the substances exercise lethal action on all these organisms. The acid-fast organism *Mycobact. phlei* is particularly sensitive to the presence of certain of them; thus one strain, in broth (p_H 6·1), is killed within 24 hours at 37° when 4-hydroxy-2 : 7-di-*n*-hexylindan-1 : 3-dione is present at a concentration of 1 : 20,000,000. Another strain of the same organism is killed under the same conditions when the same compound is present at a concentration of 1 : 11,110,000.

On the other hand these compounds have little or no adverse action on the proliferation, in broth, of the Gram-negative organisms *Bact. coli*, *Bact. pyocyaneum* and *Bact. prodigiosum*.

5. The antiseptic activities of the new substances are depressed considerably by culture media which are slightly alkaline in reaction, and are suppressed entirely by ox-serum.

REFERENCES.

Black, Shaw and Walker (1931). *J. Chem. Soc.* 272.
Churchman (1926). *Stain Technology*, **1**, 27.
Coulthard, Marshall and Pyman (1930). *J. Chem. Soc.* 280.
Dohme, Cox and Miller (1926). *J. Amer. Chem. Soc.* **48**, 1688.
Henry (1923). *Pharm. J.* 28.
Johnson and Lane (1921). *J. Amer. Chem. Soc.* **43**, 348.
Karrer and Rosenfeld (1921). *Helv. Chim. Acta*, **4**, 707.
Klarmann (1926). *J. Amer. Chem. Soc.* **48**, 2358.
—— and Figdor (1926). *J. Amer. Chem. Soc.* **48**, 791.
Leonard (1924). *J. Amer. Med. Assoc.* **83**, 2005.
Morgan, Cooper and Burtt (1923). *Biochem. J.* **17**, 30.
Nencki (1897). *Ber. deutsch. chem. Ges.* **30**, 1768.
Pyman (1927). *J. Inst. Brew.* **33**, 295.
Sandulesco and Girard (1930). *Bull. Soc. Chim.* (iv), **47**, 1300.
Walker (1924). *J. Inst. Brew.* **30**, 570, 712.
—— (1925). *J. Inst. Brew.* **31**, 562.
—— Suthers, Roe and Shaw (1931). *J. Chem. Soc.* 514.
Wieland and Marz (1926). *Ber. deutsch. chem. Ges.* **59**, 2352.
—— —— and Hoek (1925). *Ber. deutsch. chem. Ges.* **58**, 102, 2012.
Wöllmer (1925). *Ber. deutsch. chem. Ges.* **58**, 672.

CCXXVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXV. 3:5-DIHYDROXYPHTHALIC ACID, A NEW PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM BREVI-COMPACTUM* AND RELATED SPECIES.

By ALBERT EDWARD OXFORD and HAROLD RAISTRICK.

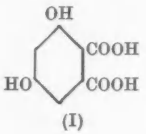
From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received October 5th, 1932.*)

In Part XXIV of this series [Clutterbuck, Oxford, Raistrick and Smith, 1932] it was shown that out of 15 species in the *P. brevi-compactum* series submitted to examination, 14 metabolised glucose into a mixture of phenolic acids from which were isolated four new substances with the empirical formulae $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$ respectively. The last-named was detected with certainty in 10 cases only, but since the yield of this substance actually isolated was never greater than 0·03 % of the sugar metabolised, being always much smaller than that of any of the other metabolic products, and since the method for its isolation and separation from tarry and other impurities inevitably led to considerable losses, it appears quite likely that it was in fact produced in all 14 instances. This paper deals with the constitution of this compound $C_8H_6O_6$, which, remarkably enough, is isomeric with puberulic acid [Part XXIII, Birkinshaw and Raistrick, 1932], a metabolic product of *P. puberulum* Bainier and *P. aurantio-virens* Biourge, the constitution of which is not yet completely elucidated, although, in view of what follows later it is of interest that these authors consider that it is probably not a dihydroxybenzenedicarboxylic acid (Part XXIII, 1932, pp. 447–9).

It was recorded in Part XXIV (p. 1455) that the new compound $C_8H_6O_6$ loses nearly 1 molecule of water (together with some CO_2) at its first M.P. (188–190°), titrates sharply as a dibasic acid, and gives a stable deep red coloration with ferric chloride and a magenta coloration with bleaching powder solution. It has since been found that complete methylation with diazomethane yields a crystalline tetramethyl derivative which is insoluble in cold alkali and gives no coloration with ferric chloride. These facts not only make it probable that $C_8H_6O_6$ is a dihydroxybenzenedicarboxylic acid, but also point clearly to one particular acid in that series, since the only dihydroxy-

benzenedicarboxylic acid which would behave as a true phthalic acid on heating and also exhibit the colour reactions of β-resorcylic acid is the unknown *3:5-dihydroxyphthalic acid* (I). The dimethyl and diethyl ethers of this acid



(I)

and their anhydrides are, however, known [Fritsch, 1897], having been synthesised by a series of reactions leaving no doubt as to their constitution. Asahina and Asano [1932] have also synthesised 3:5-dimethoxyphthalic anhydride by the same method, for comparison and identification with an oxidation product of dimethylolivetonic acid, the latter being derived, by methylation and opening of a lactone ring, from olivetonide, one of the products of the fission (by dehydration) of the lichen acid olivetoric acid extracted from the lichen *Alectoria divergens*.

The dimethyl ether of our mould metabolic product was readily obtained by hydrolysis of the tetramethyl derivative by hot alkali, and was found to crystallise from water with $1H_2O$ (lost below 100°) and to lose water of constitution at 157–158°, to yield the corresponding anhydride, M.P. 147–149°. All these facts are in complete agreement with those recorded by Fritsch [1897] for 3:5-dimethoxyphthalic acid (M.P. 158°) and anhydride (M.P. 147°). The fully ethylated derivative was an oil which lost only one ethyl group when hydrolysed by hot dilute alkali, but the resulting monoethyl ester of the diethyl ether lost the elements of ethyl alcohol at about 180° to yield an anhydride, M.P. 133–134°, a value quite close to 130°, the recorded M.P. of 3:5-diethoxyphthalic anhydride. The identity of our product $C_8H_6O_6$ with 3:5-dihydroxyphthalic acid was finally placed beyond doubt by direct comparison of the anhydride of its dimethyl ether with an authentic specimen of synthetic 3:5-dimethoxyphthalic anhydride very kindly supplied by Prof. Asahina, when a mixed melting-point showed no depression. It is of some interest that this is the first instance in which a constitutional relationship has been definitely established between a mould metabolic product and a group of lichen acids, the structure proposed by Asahina and Asano [1932, p. 477] for olivetoric acid involving the union by loss of H_2O of two fragments, each of which is a 2:4-dihydroxybenzoic acid with a long side chain in the 6-position, and theoretically convertible into 3:5-dihydroxyphthalic acid by oxidation of that side chain. Orsellinic acid, the fundamental unit in the structure of many lichen acids, *e.g.* lecanoric acid, evernic acid, gyrophoric acid, has a similar structure, the side chain in the 6-position being a methyl group. This constitutional relationship is important since it supports the view that it is the fungal part of the fungus-alga symbionts constituting the lichen which is responsible for the formation of the lichen acids.

Experimental.

Properties of the new metabolic product $C_8H_6O_6$ (3:5-dihydroxyphthalic acid).

The general properties of this compound are given in Part XXIV [Clutterbuck, Oxford, Raistrick and Smith, 1932, p. 1455] but are briefly as follows: m.p. 188–190° (efferv.) remelting at 206–210°; crystallises from ethyl acetate-light petroleum in well-formed hexagonal tablets with slow, and in flint-shaped leaflets with rapid, crystallisation. It is readily soluble in cold water and in most organic solvents except light petroleum and benzene. With aqueous or alcoholic ferric chloride it gives a stable deep red colour with perhaps a trace of purple. It gives no precipitate with bromine water. With bleaching powder solution and a dilute solution of the acid in water, a transient magenta colour is obtained. No precipitate is obtained with 2:4-dinitrophenylhydrazine in 2*N* HCl.

Dimethyl ester of the dimethyl ether (dimethyl 3:5-dimethoxyphthalate). 0·4492 g. of the pure substance, which had been recrystallised several times from ethyl acetate-light petroleum, was suspended in dry ether and treated with a large excess of diazomethane prepared from nitrosomethylurethane (4·2 cc.). A vigorous effervescence took place and the solution was allowed to stand for several hours. A few drops were then tested for free phenols by dilution with alcohol and addition of a few drops of dilute ferric chloride solution, when a moderately intense but transient purple coloration was obtained. A further quantity of ethereal diazomethane was therefore added (from 5 cc. nitrosomethylurethane) and the reaction mixture allowed to stand for 2 hours, the ferric chloride test being then negative. The ether was evaporated off and the colourless, crystalline residue (0·6145 g.) was recrystallised from aqueous alcohol, giving colourless, hair-fine needles, m.p. 92–94°, having a very pronounced ethereal odour.

Micro-analysis (Schoeller): loss at 80° in high vacuum (probably due to slow sublimation) 7·54, 5·87 %. Found on material dried at 80° in high vacuum: C, 56·65, 56·76 %; H, 5·64, 5·71 %; OCH_3, 47·18, 48·08 %; mol. wt. 250, 268. $C_8H_2O_2(OCH_3)_4$ requires C, 56·67 %; H, 5·55 %; OCH_3, 48·83 %; mol. wt. 254.

Dimethyl ether (3:5-dimethoxyphthalic acid). The crude tetramethyl derivative (0·43 g.) was hydrolysed by suspending it in a mixture of 20·0 cc. *N* NaOH and 20 cc. ethyl alcohol and boiling the whole under reflux for 1 hour. The undissolved part (0·03 g.) was then filtered off, and the filtrate exactly neutralised by *N* H_2SO_4 (16·6 cc.), indicating that 3·4 cc. of *N* NaOH had been used up (theoretical, 3·15 cc.). More sulphuric acid was then added and the liquid extracted several times with ether. Evaporation of the dried extract yielded a crystalline deposit which crystallised from boiling water (charcoal) in nearly colourless pointed prisms (0·15 g.), m.p. 157–158° (efferv.), with slight softening previously at about 100°. The melt was taken to 170° and maintained at this temperature till effervescence ceased. It reset immediately on removal from the bath and then re-melted at 143–148°.

Micro-analysis (Schoeller): On hydrated material; C, 49·44, 49·21 %; H, 5·17, 4·98 %; loss at 80° *in vacuo*, 7·45 %. $C_8H_4O_4(OCH_3)_2$, H_2O requires C, 49·16 %; H, 4·96 %; H_2O 7·38 %. On material dried at 80° *in vacuo*: C, 52·44, 52·22 %; H, 4·81, 4·61 %; OCH_3, 27·41, 28·24 %. $C_8H_4O_4(OCH_3)_2$ requires C, 53·08 %; H, 4·45 %; OCH_3, 27·41 %.

0·1474 g. of the hydrated material required 12·17 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 121, the theoretical for $C_8H_4O_4(OCH_3)_2$, H_2O titrating as a dibasic acid, being 122. This ether gives no coloration with ferric chloride either in aqueous or alcoholic solution. It is moderately soluble in cold water and is so readily soluble in cold alcohol that purification by crystallisation from aqueous alcohol, as recommended by Fritsch [1897] leads to considerable loss of material.

Anhydride of the dimethyl ether (*3:5-dimethoxyphthalic anhydride*). The above ether was heated at 170–180° in an oil-bath until effervescence ceased, and the residue purified by sublimation *in vacuo*. The sublimate (micro-needles) melted at 147–149°, and when mixed with a specimen of authentic 3:5-dimethoxyphthalic anhydride, supplied by Prof. Asahina and resublimed by us (M.P. 146–148°) had M.P. 146–149°. Several determinations of the mixed M.P. were carried out.

Micro-analysis (Schoeller): C, 57·64, 57·70 %; H, 3·79, 3·96 %; OCH_3, 29·84, 29·79 %. $C_8H_2O_3(OCH_3)_2$ requires C, 57·66 %; H, 3·87 %; OCH_3, 29·80 %.

Monoethyl ester of the diethyl ether (*monoethyl 3:5-diethoxyphthalate*). 0·5 g. $C_8H_6O_6$ were ethylated by exactly the same procedure given above for methylation, except that nitrosoethylurethane was used in place of nitrosomethylurethane. The product (1·06 g.) was a yellow syrup not completely soluble in alcohol, the alcohol-insoluble portion, a colourless amorphous solid, M.P. 70–80°, being readily soluble in light petroleum. The analytical figures for this latter product correspond closely with those for $(CH_2)_n$, and the hydrocarbon was doubtless formed by polymerisation and decomposition of diazoethane.

Micro-analysis (Schoeller): C, 84·8 %; H, 14·5 %; $(CH_2)_n$ requires C, 85·7%; H, 14·3 %.

The alcohol-soluble ethylation product was hydrolysed by boiling with *N*/2 aqueous-alcoholic NaOH until nearly all had gone into solution. The mixture was then cooled, filtered, acidified with dilute H_2SO_4 and extracted with ether, the extract being dried over anhydrous Na_2SO_4. Evaporation of the ether yielded a yellow, semi-solid paste, which after two recrystallisations from boiling water yielded colourless, long, silky needles, M.P. 97–99° with no effervescence (according to Fritsch 3:5-diethoxyphthalic acid melts at 182° with effervescence). The substance is soluble in cold sodium bicarbonate solution, and gives no coloration with ferric chloride. The following analytical figures (Schoeller) show conclusively that it is a triethyl derivative of $C_8H_6O_6$. Found: C, 59·57 %; H, 6·50 %; OC_2H_5, 47·06 %; $C_8H_3O_3(OC_2H_5)_3$ requires C, 59·55 %; H, 6·42 %; OC_2H_5, 47·88 %.

Anhydride of the diethyl ether (3:5-diethoxyphthalic anhydride). The above monoethyl ester showed no tendency to decompose at 140°, but when heated at 180° vigorous effervescence took place, and the odour of ethyl alcohol was perceptible. Heating was continued for 15 minutes after effervescence had ceased, and the residue was sublimed *in vacuo*. The crystalline sublimate had M.P. 133–134°, was insoluble in cold sodium bicarbonate solution, and not very readily soluble in cold caustic potash solution.

Micro-analysis (Schoeller): C, 60·92 %; H, 5·41 %; OC_2H_5, 39·35 %; $C_8H_2O_3(OC_2H_5)_2$ requires C, 60·99 %; H, 5·13 %; OC_2H_5, 38·16 %.

Attempt to isolate 3:5-dihydroxyphthalic anhydride. Pure $C_8H_6O_6$ (0·4 g.) was heated in a stream of nitrogen at 190–200° until a quiet melt was obtained and no more water or CO_2 was evolved. A trace of material had sublimed and condensed on the cool part of the tube. The solid residue, dark in colour, was readily and completely soluble in saturated $NaHCO_3$ solution. This was immediately shaken with ether but it was found that practically nothing was extractable. The solution was acidified and again extracted with ether, the ether extract yielding a solid product which, after crystallisation from chloroform containing a little ethyl acetate, was obtained in the form of needles melting between 210 and 220° with marked softening previously at 190°, and having an equivalent of about 120. The product gave an intense purple-red colour with ferric chloride and was probably a mixture of the original $C_8H_6O_6$ together with α-resorcylic acid, but a separation was not possible on account of the small quantity of material available.

Summary.

The new mould metabolic product $C_8H_6O_6$ (M.P. 188–190°) formed from glucose by species in the *P. brevi-compactum* series, has been identified as 3:5-dihydroxyphthalic acid, and hence is constitutionally related to a large group of lichen acids.

REFERENCES.

Asahina and Asano (1932). *Ber. deutsch. chem. Ges.* **65**, 475.
Birkinshaw and Raistrick (1932). *Biochem. J.* **26**, 441.
Clutterbuck, Oxford, Raistrick and Smith (1932). *Biochem. J.* **26**, 1441.
Fritsch (1897). *Liebig's Ann.* **296**, 344.

CCXXVII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXVI. THE FORMATION FROM GLUCOSE BY MEMBERS OF THE *PENICILLIUM CHRYSOGENUM* SERIES OF A PIGMENT, AN ALKALI-SOLUBLE PROTEIN AND PENICILLIN—THE ANTIBACTERIAL SUBSTANCE OF FLEMING.

By PERCIVAL WALTER CLUTTERBUCK, REGINALD LOVELL and HAROLD RAISTRICK.

From the Divisions of Biochemistry and Bacteriology, London School of Hygiene and Tropical Medicine, University of London.

(*Received October 14th, 1932.*)

In a recent paper, Fleming [1929] showed that a mould which appeared as an air contaminant in laboratory bacterial cultures, and which resembled *Penicillium rubrum* Grasberger-Stoll, produced when grown on nutrient broth a very powerful antibacterial substance. Filtrates of the broth cultures were named "penicillin" and it was found that the active substance affected different bacteria in different degrees. The action was very marked on the pyogenic cocci and the diphtheria group of bacilli, whilst the coli-typhoid and the influenza bacillus groups were quite insensitive to it. Staphylococci, *Streptococcus pyogenes* and the pneumococcus were completely inhibited in a 1 in 800 dilution of the filtrate, which did not affect the growth of *H. influenzae* (Pfeiffer's bacillus), and a method was described for the isolation of *H. influenzae* by incorporating penicillin in a suitable solid medium.

Examination of Fleming's organism by Dr Charles Thom at our request showed that it was not *Penicillium rubrum* but a strain closely related to *P. notatum* Westling in the *P. chrysogenum* Thom series. Dr Thom writes as follows: "I have cultivated Fleming's organism under several different conditions and cannot agree with the nomenclature as *P. rubrum* either in the sense of my 1910 paper or in the sense of Biourge's monograph. In fact, I believe this culture, although showing some divergencies in culture reactions, to be closer to *P. notatum* of p. 264 in my book than to the groups discovered on pp. 249–50 as indicated by the nomenclature used."

In his book [1930, pp. 261–2] Dr Thom says that the type strain of *P. chrysogenum* (No. 26) rapidly digests milk and liquefies gelatin giving a yellow colour. "Studied on a series of culture media, it changes colour with the composition of the substratum and with the age or stage of metabolism

of the colony itself: colonies grown on Czapek's solution in presence of abundance of fermentable sugar long remain green, without such sugar little or no yellow appears in the substratum." The production of this yellow pigment on milk appears to be characteristic of species in the *P. chrysogenum* series. One of the results of this work was the isolation and investigation of this pigment to which the name chrysogenin is given.

Although Fleming's organism appears, on the usual systematic criteria and from the fact that it produces chrysogenin, to belong to the *P. chrysogenum* series, the fact (see below) that we have never been able to demonstrate the production of the bactericidal substance, penicillin, by *P. chrysogenum* or by *P. notatum* renders the identification of Fleming's organism with either of these species doubtful from a physiological point of view.

Working both with Fleming's strain and the type strain of *P. chrysogenum* (No. 26), we have frequently experienced difficulty in repeating our experiments. Thus single spore cultures gave colonies sometimes with a white and sometimes with a yellow reverse, and when used for sowing flasks of our medium the former gave greater yields of chrysogenin than the latter. Moreover, with both strains after continued subcultivation on our medium, the mycelial felts tended to become less green, to show white patches, floccose in character, and this was always accompanied by decrease in the production of both chrysogenin and penicillin. From such cultures, by single spore technique, both floccose and green colonies were re-obtained, and the latter, but not the former, on using for sowing gave considerable chrysogenin formation (with all the strains) and penicillin formation (with Fleming's strain).

Eight cultures, for which we are indebted to our colleagues Miss L. Farrell and the late Mr J. H. V. Charles, have thus been employed.

Catalogue No.	Strain	Source
589	*P. chrysogenum* Thom (No. 26)	British National Collection of Type Cultures, Lister Institute
P 19 W	,, white reverse	Isolated from culture No. 589 at the London School of Hygiene and Tropical Medicine
P 19 Y	,, yellow reverse	Do.
P 19 Y_2	,, white floccose form	Do.
57	Fleming's strain (*a*)	British National Collection of Type Cultures, Lister Institute
152	,, (*b*)	Dr Fleming
I.D.	Fleming's strain, white floccose form	Isolated from culture No. 57 at the London School of Hygiene and Tropical Medicine
P 45	*P. notatum* Westling	Dr C. Thom

With all these strains (except the two giving a floccose type of mycelium, Nos. P 19 Y_2 and I.D.), the metabolism solutions gradually assumed a yellow colour and a negative rotation and gave a characteristic olive-brown colour with ferric chloride. The metabolism solutions on acidifying to p_H 3·5 gave a

yellow flocculent precipitate which was shown to consist chiefly of the yellow pigment chrysogenin, and a protein. Only the two green strains, cultures Nos. 57 and 152, and to a much smaller extent, the floccose culture I.D. from Fleming's culture, showed any antibacterial activity.

This activity, although arising more slowly on this synthetic medium, was as great or greater than that recorded by Fleming using a broth medium. This should be of considerable practical importance since the isolation of penicillin from a synthetic medium should be much simpler than from broth.

The purpose of the present paper is to describe the isolation of the pigment chrysogenin, and the protein, using the type strain of *P. chrysogenum* Thom No. 26, and the preliminary attempts at isolation of penicillin, using Fleming's culture.

Preparation of the three chief metabolic products.

A quantity of modified Czapek-Dox medium was made up having the following composition, for which we are indebted to our colleague Dr H. G. Turley:

$NaNO_3$	3·0 g.
KH_2PO_4	1·0 g.
KCl	0·5 g.
$MgSO_4, 7H_2O$	0·5 g.
$FeSO_4, 7H_2O$	0·01 g.
Glucose	40·0 g.
Distilled water to 1000 cc.	

400 cc. quantities of this medium were placed in 100 1-litre conical flasks which were plugged with cotton wool, and sterilised by steaming for half an hour on each of three consecutive days. Each flask was heavily sown with a suspension in the above medium of spores of a 6-day culture of either the type strain of *P. chrysogenum* or of Fleming's organism, grown on Czapek-Dox agar at 25°. The flasks were shaken and then incubated at 25° for 21–28 days, until the sugar had almost entirely disappeared, when a sample of the filtered metabolism medium from the type strain of *P. chrysogenum* gave in a 2 dm. tube a rotation of − 0·3 to − 0·5°. With Fleming's strain the rotation with optimum antibacterial activity was − 0·03 to − 0·08° but increased to − 0·36° during the subsequent few days.

The contents of the flasks were now filtered, the mycelium pressed and the combined filtrates (about 38 litres) were acidified to p_H 3·6 with about 480 cc. 2*N* H_2SO_4. With careful acidification a yellow precipitate was obtained which quickly flocculated, but if excess of acid was rapidly added the precipitate remained in an unfiltrable dispersed form. It was found with each organism that the crude precipitate consisted of about equal weights of protein and chrysogenin. In the case of Fleming's organism this precipitate could be filtered off almost without loss of antibacterial activity. A further small amount of chrysogenin can be precipitated from the filtrate by saturating

with salt and filtering. From a typical batch of 100 flasks (using the type strain of *P. chrysogenum*) 13 g. of crude precipitate were obtained containing 4·2 % of nitrogen by Kjeldahl. This material was then extracted exhaustively with ether in a Soxhlet apparatus and was thus separated into two fractions: (*a*) an ether-insoluble fraction containing 8·53 % N, (*b*) an ether-soluble pigment containing only traces of N.

Purification of the nitrogenous substance (protein).

The crude nitrogenous substance readily dissolved in dilute NaOH and could be roughly titrated by adding excess *N*/10 alkali and back-titrating using phenolphthalein as indicator. Thus 0·1903 g. of the crude material required 4·3 cc. *N*/10 NaOH. For purification, 7 g. of the substance were dissolved in excess dilute (160 cc. *N*/10) NaOH, diluted to 300 cc. and 200 cc. of absolute alcohol added. After filtering, 16 cc. of *N* HCl were added with vigorous shaking and the solution centrifuged. The supernatant highly pigmented layer was poured off. The process was repeated. The protein was then repeatedly shaken with 50 % alcohol (increasing gradually to absolute) and again centrifuged until all the colouring matter had been removed. The material was finally pressed on a Büchner funnel, washed with alcohol and ether and dried. The dried residue weighed 4·1 g., and had an ash content (Schoeller) of 0·54 %. The following figures were obtained by micro-combustion (Schoeller): C, 51·51, 51·76 %; H, 6·85, 6·95 %; N, 12·75, 12·95 %; S, 1·35, 1·32 %. A macro N determination also gave 12·95 % and this figure was used for the subsequent Van Slyke analysis of the protein.

0·3613 g. of this purified material required 3·6 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 1004.

Van Slyke analysis of the nitrogenous product (protein).

The above figures suggest that the substance is either a polypeptide or protein and since, so far as we are aware, no similar nitrogenous product has been isolated from mould metabolism solutions, a detailed study of the nature of the product was embarked on, by means of the Van Slyke nitrogen distribution method [1911; 1915].

Hydrolysis of nitrogenous product. 3·3439 g. of purified and dried product (containing 12·95 % N and 0·433 g. of total N) were boiled in a tared flask under reflux with 61·1620 g. of 20 % hydrochloric acid for 6 hours, the flask being heated in an oil-bath at 130–135°. The hydrolysis was then stopped, the flask cooled and its weight adjusted to the original weight by addition of water.

1 cc. of the fluid was then removed, made up to 10 cc. in a graduated flask and retained for the amino-nitrogen determination by the Van Slyke method. The remainder was re-weighed and the hydrolysis continued for a further period of 3 hours. The hydrolysis was then stopped, the weight adjusted, a

1 cc. sample removed and made up to 10 cc. for the amino-nitrogen determination as before, and the residue re-weighed.

Analysis of hydrolysis fluid. A small amount (2 cc.) of the diluted solution after 6 and 9 hours' hydrolysis gave 2·14 and 2·13 cc. amino-nitrogen respectively. It appeared therefore that the protein was readily hydrolysed, the hydrolysis being complete in 6 hours or less. In two other experiments the hydrolysis was almost complete in the same time using $2N$ H_2SO_4 instead of 20 % HCl. The amino-nitrogen figure (2·14 cc.) corresponds with a total amino-nitrogen of 0·3441 g., and the ratio after hydrolysis of amino/total nitrogen = 79·45 %. After removal of the samples for these determinations, the residue calculated on a weight basis corresponded with the presence of 3·224 g. hydrolysed protein or a total N content of 0·4176 g. This solution of hydrolysed protein was washed into a Claisen flask and evaporated to dryness *in vacuo* at 45°. Water was added and the evaporation continued, thus removing most of the hydrochloric acid. The residue was then dissolved in water and made up to 250 cc.

The total N was determined on two samples, each of 15 cc., by Kjeldahl and was 0·4176 g., agreeing exactly with the value obtained by calculation on a weight basis.

After removal of these samples, the residual solution corresponded to 2·837 g. of hydrolysed protein or a total N content of 0·3675 g., and the remaining determinations were made on this solution.

Ammonia was first determined in the hydrolysate by distillation with lime under reduced pressure. The melanin formed during hydrolysis and absorbed on the excess lime during the ammonia determination was filtered off and the nitrogen content obtained by Kjeldahl. The bases were then precipitated with purified phosphotungstic acid, filtered and washed, following the modified technique of Hartley [1914] and Van Slyke [1915]. The phosphotungstic acid precipitate of the bases was then decomposed by the hydrochloric acid-amyl alcohol-ether method [Van Slyke, 1915], the arginine being determined in the usual way, and the cystine by the Denis modification [1910] of the Benedict method.

Results. Ammonia. The amount of $N/10$ acid neutralised was 17·6 cc. indicating 0·0246 g. amide-nitrogen = 6·69 % of the total N.

Melanin. The amount of $N/10$ acid neutralised was 11·7 cc. indicating 0·0164 g. melanin-nitrogen = 4·46 % of the total N.

Cystine. The $BaSO_4$ weighed 0·043 g. indicating 0·0129 g. cystine-nitrogen = 3·51 % of the total N.

Arginine. The amount of $N/10$ acid neutralised was 5·8 cc. indicating 0·0324 g. arginine-nitrogen. Applying the cystine correction this reduces to 0·0302 g. arginine-nitrogen = 8·22 % of the total N.

Total nitrogen of bases. The amount of $N/10$ acid neutralised in the Kjeldahl determinations was 23·5 cc. Added to the amounts neutralised by the ammonia evolved in the arginine determination (5·8 cc.) the total acid

neutralised = 29·3 cc. indicating a total N of bases of 0·0820 g. N = 22·31 % of the total N.

Amino-nitrogen of bases. The mean amount of N gas evolved with 2 cc. (of 50 cc.) of solution was 2·73 cc. at 20° and 754 mm. indicating a total amino-nitrogen of bases of 0·0402 g. = 10·93 %.

Total nitrogen of filtrate from bases. The mean amount of $N/10$ acid neutralised per 25 cc. (of 150 cc.) was 29·5 cc. indicating 0·2478 g. N = 67·42 % of the total N.

Amino-nitrogen of filtrate from bases. The mean amount of nitrogen gas evolved with 2 cc. (of 150 cc.) was 5·3 cc. at 20° and 754 mm. indicating a total amino-nitrogen of filtrate of 0·2282 g. = 62·09 % of the total N.

The results are summarised in Table I.

Table I. *Results of protein analysis in percentages of total nitrogen.*

	Found g.	% of total nitrogen	Corrected for solubility of bases g.
Ammonia-N	0·0246	6·69	—
Melanin-N	0·0164	4·46	—
Cystine-N	0·0129	3·51	0·0155
Arginine-N	0·0302	8·22	0·0334
Histidine-N	0·0313	8·51	0·0351
Lysine-N	0·0076	2·07	0·0081
Amino-N of filtrate from bases	0·2282	62·09	0·2230
Non-amino-N of filtrate from bases	0·0196	5·33	0·0147
Total N	0·3708	100·88	—

The mould protein appears therefore to resemble fairly closely a typical alkali-soluble leaf protein. The following table gives the corresponding figures for the mould protein and the alkali-soluble protein of the alfalfa leaf [Dowell and Menaul, 1921].

Nitrogen content	Protein of alfalfa leaf %	Mould protein %
Total nitrogen	13·6	12·95
NH_3-N	6·8	6·69
Melanin-N	7·8	4·46
Arginine-N	11·01	8·22
Histidine-N	6·26	8·51
Cystine-N	0·85	3·51
Lysine-N	5·26	2·07
Monoamino-N	53·53	62·09
Non-amino-N	8·48	5·33

Action of trypsin and pepsin on the nitrogenous product.

The protein nature of the nitrogenous product was finally confirmed by its hydrolysis by trypsin in alkaline and by pepsin in acid medium.

A solution of 1 % protein in 0·5 % Na_2CO_3 was incubated at 37° after addition of trypsin along with controls containing on the one hand similar amounts of protein and carbonate, and on the other of trypsin and carbonate. 1 cc. of solution was removed for determination of amino-nitrogen by Van Slyke's method. The increase of amino-nitrogen (as cc. of nitrogen gas) per cc. of fluid due to the action of trypsin on the protein was from the blank value

of 0·38 cc. nitrogen to 1·08 cc. after 20 hours, and 1·40 cc. after 48 hours. A similar series of values was obtained from the action of pepsin in *N*/30 HCl, but the action was much slower. Thus the blank value of 0·43 cc. nitrogen increased to 0·75 cc. after 48 hours and to 1·05 cc. after 96 hours.

Isolation of chrysogenin.

The ether-soluble pigment chrysogenin, on removal of the ether was obtained as a transparent glaze on the side of the containing vessel. It was extremely soluble in most organic solvents except light petroleum in which it was insoluble. Attempts to crystallise it by slow evaporation of mixtures of solvents failed, the material always separating on the vessel as a glaze. It could, however, be precipitated from ether solution by light petroleum as a non-crystalline light yellow powder. This powder consisted chiefly of chrysogenin, together with a small amount of a reddish-brown dehydration product, the pigment readily losing water to form this compound. The dehydrated product was, however, not very soluble in benzene, and could be separated by shaking the yellow powder with benzene, filtering and reprecipitating the pigment with light petroleum. After repeating this treatment the product from several batches appeared homogeneous and gave the same analytical figures.

The following values were obtained on micro-combustion (Schoeller): C, 64·75, 65·06 %; H, 6·58, 6·51 %; OCH_3, nil. $C_{18}H_{22}O_6$ requires C, 64·69 %; H, 6·58 %.

This purified preparation was obtained in micro-crystalline form by dissolving in a very slight excess of *N*/10 NaOH and precipitating by passing a stream of CO_2. After precipitating in this way twice, filtering, washing and drying, a sample gave the following figures on micro-combustion (Schoeller): C, 64·82, 64·84 %; H, 6·52, 6·54 %; $C_{18}H_{22}O_6$ requires C, 64·69 %; H, 6·58 %.

The analytical figures agree very closely amongst others with those required by the formulae $C_{15}H_{18}O_5$, $C_{18}H_{22}O_6$. The molecular weight (Schoeller) was returned as 470, 490, but the results of titration and preliminary methylation experiments favour the formula $C_{18}H_{22}O_6$ (mol. wt., 334) and in view of possible errors in molecular weight determination by the Rast camphor method this latter value is provisionally adopted.

Properties of chrysogenin.

Chrysogenin is very soluble in ether, benzene, acetone, ethyl alcohol, ethyl acetate and chloroform, but insoluble in light petroleum and water. It gives a characteristic olive-brown colour with $FeCl_3$. It is very strongly *l*-rotatory, the specific rotation being $[\alpha]_{5461} - 762°$, $[\alpha]_{5790} - 570°$, and it is thus responsible for the development of the negative rotation in the metabolism medium. It possesses two very weakly acidic groups, and probably does not contain a free carboxyl group. Thus a solution containing 0·1320 g. of pigment, on

titration with $N/10$ NaOH to phenolphthalein, became more and more deeply reddish-brown until it was impossible to see the indicator colour, and the titration was continued using the indicator externally. After addition of 4·0 cc. $N/10$ NaOH (corresponding with an equivalent of 330, $C_{18}H_{22}O_6$ requires 334) the colour of the solution suddenly changed again from dark reddish-brown to light yellow. Further addition of NaOH led to a second increase of the red-brown colour and the end-point came after an addition of 8·22 cc. (corresponding to an equivalent of 160·6, $C_{18}H_{22}O_6$ assumed dibasic requires 167). On passing CO_2 through this neutralised solution the pigment again separated in microcrystalline form. Electrometric titration, kindly carried out by Dr Kekwick in Prof. Drummond's laboratory, showed that two stages of buffering were present, but the groups responsible were so weakly acidic that reliable dissociation constants could not be obtained, and only a rough value, 193, for the lower equivalent was given. The absence of a carboxyl group is also indicated by the fact that the substance does not esterify with ethereal diazomethane.

Chrysogenin on warming with dilute NaOH gives amongst other unidentified products acetaldehyde and sorbic acid. It does not methylate with ether-diazomethane, but gives with ether-nitrobenzene-diazomethane a monomethyl derivative which no longer gives the characteristic olive-brown colour with $FeCl_3$. The pigment reduces readily with Zn and HCl giving a colourless compound dihydrochrysogenin, $C_{18}H_{24}O_6$ (found: C 64·48 %; H 7·10 %; $C_{18}H_{24}O_6$ requires C 64·27 %; H 7·20 %) which readily re-oxidises in air, gives a wine-red colour with $FeCl_3$, and with ether-diazomethane gives a dimethyl derivative which shows a green colour with $FeCl_3$. The above facts suggest that chrysogenin contains a quinol nucleus. Investigation of its structure is reserved for a separate communication.

Antibacterial activity of the metabolism fluid of Fleming's organism.

A few preliminary experiments confirmed the observations of Fleming [1929] that *Bact. coli* and *H. influenzae* were resistant to the action of "penicillin" whilst *Staph. albus*, *Strept. haemolyticus*, *Strept. pneumoniae* and *N. meningitidis* were susceptible. The pneumococcus (*Strept. pneumoniae*) and Pfeiffer's influenza bacillus (*H. influenzae*) were selected for further experiments, the latter being used as a control.

Technique for determination of antibacterial power.

The metabolism fluid, after passing through a Seitz filter, was incorporated in both fluid and solid media.

Fluid media. Dilutions of the filtrate from 1/10 to 1/5120 falling in geometrical progression were made, using sterile precautions, in 1 cc. quantities of the test media. For the pneumococcus, Hartley's trypsinised horse muscle

broth was used, and for the influenza bacillus heart broth to which had been added 5 % of Fildes's extract [Fildes, 1920]. In each case a control tube was included containing the test media without the addition of metabolism solution.

After the preparation of the media, all the tubes were seeded with one loopful (approx. 1/250 cc.) of an 18 hours' broth culture of the test organism. All were then incubated at 37° for 24 hours and opacity readings taken. No difference between the early and late readings was apparent in a few experiments in which incubation was prolonged for 48 and 72 hours.

Solid media. In each experiment Petri dishes containing 15 cc. of solid media with the metabolism solution in a concentration of 1 in 20 were included, as a check on the detailed titrations in fluid media. The medium used for the pneumococcus was 5 % horse blood agar, and for the influenza bacillus 5 % Fildes's agar. Controls containing no metabolism solution were always included. The plates were streaked with one loopful of the test cultures.

Results of experiments.

The maximum yield of antibacterial substance produced by Fleming's culture in a broth medium appeared about the eighth day, which corresponds with the findings of Fleming. On the other hand, the yield in the synthetic medium was greater and reached its maximum concentration about the 16th to the 20th day. Further incubation resulted at first in a slight loss and later in a rapid loss of antibacterial activity. The following table gives the result of testing the yield over a short period.

Period of growth of Fleming's culture	Dilution of metabolism fluid inhibiting growth of	
	Strept. pneumoniae	*H. influenzae*
15 days	1/640	1/10
16 „	1/1280	1/10
18 „	1/640	—
22 „	1/640	1/10
24 „	1/160	1/10
31 „	1/160	1/10

In each experiment the control tubes gave a good growth of the test organism. On solid media, the pneumococcus was completely, and the influenza bacillus not at all inhibited at 1/20. It should be noted that this inhibition of *H. influenzae* in low dilutions occurred in relatively few experiments.

All subsequent work was carried out with filtrates from the growth of Fleming's culture on synthetic media.

Storage of filtrate.

Untreated filtrates taken when showing a high concentration of the antibacterial substance can be stored for quite long periods without great loss. A filtrate active against the pneumococcus in a dilution of 1/1280 was stored

in the ice-chest at 0° for 16 weeks without taking precautions as to sterility. Table II shows the reduction observed during that period.

Table II.

Period of storage in ice-chest at 0°	Dilution inhibiting growth of *Strept. pneumoniae*
Before storage	1 in 1280
After 5 days	1 in 1280
„ 7 „	1 in 640
„ 13 „	1 in 640
„ 14 „	1 in 640
„ 21 „	1 in 640
„ 12 weeks	1 in 320
„ 16 „	1 in 320

This method of storage was, however, inadequate as in two experiments the fluid became infected and antibacterial power was immediately lost. When the fluid was adjusted to p_H 5–6, passed through a sterilised Seitz filter into sterile flasks, and stored at 0°, the antibacterial power remained unchanged for 3 months. In all the experiments the p_H was readjusted to 7·2 before testing for antibacterial activity.

Antibacterial inactivity of chrysogenin and protein.

It has already been shown that careful adjustment of the p_H of the filtered metabolism solution to 3·6 caused the flocculation of most of the protein together with a large portion of chrysogenin. The filtrate, after removal of this precipitate and adjustment of the p_H to 7·2 still contained most of the antibacterial substance, whilst the precipitate on redissolving in dilute NaOH and readjusting the p_H showed no activity at all. Samples of dihydrochrysogenin, purified chrysogenin and protein also showed no activity.

Effect of evaporation of the metabolism fluid on the antibacterial power.

In order to deal successfully with the large bulk of fluid it was necessary to evaporate the fluid without serious loss of activity. Evaporation *in vacuo* at 40–45° of the filtered metabolism fluid to 1/20 its volume at p_H 2 and 3·5, with subsequent readjustment of volume and p_H resulted in complete loss of antibacterial power. Evaporation at p_H 7·2 also led to a fall of inhibitory power from a dilution of 1:1280 to 1:80. Between p_H 5 and 6, however, there appeared to be a region of greater stability and loss of activity was only small (1/640 to 1/320). It was, therefore, possible to adjust the filtered metabolism solution to p_H 3·6, to filter off most of the chrysogenin and protein, to readjust to p_H 5·5 and to evaporate *in vacuo* at this p_H, without great loss of activity.

Extraction of the antibacterial substance with ether.

Fleming regarded penicillin as insoluble in ether, but this was due to the fact that his filtrates were alkaline in reaction.

Extraction with ether at p_H 7·2 partly, and at p_H 2 completely, removes the antibacterial substance. Thus when the metabolism solution (150 cc.) was

extracted three times with ether (100 cc.) at p_H 7·2, the inhibitory power after removal of ether *in vacuo* at 40–45° fell from 1/1280 to 1/320, and after extraction at p_H 2 from 1/1280 to 1/10. When the ether of the ethereal extracts was removed in a current of air, the residue was inactive, but if the ethereal extracts together with 50 cc. water were evaporated *in vacuo* at 40–45°, the residual aqueous phase, after adjusting its volume and p_H showed a considerable portion of the original activity. Thus a solution originally inhibiting the growth of the pneumococcus in a dilution of 1/640 gave an extract showing inhibition at 1/160 (± 1/320).

From the above experiments it appears that the antibacterial substance is not identical with either chrysogenin or the protein, and that the greater part of these substances can be removed by suitable adjustment of p_H and filtering without serious loss of antibacterial activity. The antibacterial substance is extremely labile, becoming inactivated during evaporation of an ether solution in a stream of air and by evaporation *in vacuo* at 40–45° in both fairly acid and alkaline solutions. It is, however, more stable at p_H 5–6 and may be extracted with ether from acid solution. The investigation of the isolation and chemical nature of penicillin is being continued.

Summary.

The production, in synthetic media containing glucose and mineral salts with sodium nitrate as a source of nitrogen, of a new yellow pigment, chrysogenin, a new alkali-soluble protein and Fleming's antibacterial substance "penicillin" by several strains in the *P. chrysogenum* series is investigated.

Chrysogenin and the alkali-soluble protein are produced by normal strains of *P. chrysogenum* Thom, *P. notatum* Westling and by Fleming's organism. Neither of these substances is produced by the floccose forms of these organisms. Penicillin is produced only by Fleming's organism and not by the type strains of *P. chrysogenum* Thom and *P. notatum* Westling.

Chrysogenin, $[\alpha]_{5461} - 762°$, is responsible for the *l*-rotation and the olive-brown $FeCl_3$ reaction of the metabolism solution. It contains two very weakly acidic groups and is probably a quinol. The empirical formula $C_{18}H_{22}O_6$ is provisionally adopted. It does not show any antibacterial activity.

The protein is readily soluble in dilute alkali and a Van Slyke analysis shows that it closely resembles a typical alkali-soluble leaf protein. It does not show any antibacterial activity. This protein has thus been synthesised by the fungi from glucose as the sole source of carbon, and sodium nitrate as sole source of nitrogen.

Penicillin, previously obtained by growth of Fleming's species of *Penicillium* only in broth cultures, has now been prepared by growing this organism in a synthetic medium consisting solely of glucose and inorganic salts, thus permitting the hope of readier isolation. A technique for following the antibacterial power is described, and by its means the conditions for storage and

evaporation of active solutions without serious loss of activity, for separation of most of the pigment and protein and for extraction of the active material with ether, are investigated. Penicillin is an extremely labile substance, very readily inactivated by oxidation, evaporation *in vacuo* at 40–45° in both acid (p_H 2 and 3·5) and alkaline (p_H 8) solutions, but is moderately stable at p_H 5–6.

REFERENCES.

Denis (1910). *J. Biol. Chem.* **8**, 501.
Dowell and Menaul (1921). *J. Biol. Chem.* **46**, 437.
Fildes (1920). *Brit. J. Exp. Path.* **1**, 129.
Fleming (1929). *Brit. J. Exp. Path.* **10**, 226.
Hartley (1914). *Biochem. J.* **8**, 541.
Thom (1930). The Penicillia. (Baillière, Tindall and Cox, London.)
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.
—— (1915). *J. Biol. Chem.* **22**, 281.

CCXXVIII. THE BIOLOGICAL VALUES OF PROTEINS.

III. A FURTHER NOTE ON THE METHOD USED TO MEASURE THE NITROGENOUS EXCHANGE OF RATS.

By MARGARET AVERIL BOAS FIXSEN
and HESTER MARY JACKSON.

From the Division of Nutrition, Lister Institute, London.

(*Received October 12th, 1932.*)

In the preceding papers of this series [Chick and Roscoe, 1930; Boas Fixsen, 1930] a description was given of the method used in this laboratory to estimate the biological values of proteins by the balance sheet method, using adult rats as experimental animals. Experience obtained subsequently in carrying out a large number of experiments has led to the introduction of certain modifications which seem worthy of note.

Technique.

In any experiment designed to investigate the value of a given protein for replacing the daily loss of body nitrogen, it is essential to secure for the experimental animal an adequate intake of calories derived from fat and carbohydrate in order to prevent the use of the food protein to provide fuel. Hence all experiments are discarded in which the daily intake of calories from fat and carbohydrate falls below 120 kg.-cal. (per 1000 g. body wt.) for rats below 375 g. in weight, and below 115 kg.-cal. for heavier rats. These figures have been arbitrarily selected after prolonged experience.

Every effort is made to secure a constant level of food intake during the experimental period of 4 days. The results of tests in which the weight of food consumed varied by more than 1 g. on any of the 4 days have not been used for calculations of the biological values of the proteins concerned.

During the periods when nitrogen-free diet is given in order to estimate the daily loss of endogenous nitrogen, it has not been found necessary to secure a large or uniform intake of food. No relationship has been found to exist between the output of endogenous nitrogen and the intake of food (see Table I). The probable explanation of this fact is that rats of the size used in this work (300–500 g.) have sufficient reserves of body fat and carbohydrate to supply their energy requirements during this period and need not draw upon their body protein, even when the food intake is small. The loss in body weight (av. 6 g. daily) was very small in comparison with the weight of the animal.

Table I. *Results of experiments in which N-free diet was given to adult rats.*

All figures are average daily amounts.

Rat No.	Date of exp.	Average body weight g.	Intake of dry food g.	Loss of body weight during exp. g.	Nitrogen in faeces mg.	Faecal nitrogen per 100 g. dry food mg.	Nitrogen in urine mg.	Total nitrogen output mg.
18	28. xi. 30	516	15·2	6	23·4	154·0	82·9	106·3
	15. i. 31	529	6·4	6	19·7	307·4	130·6	150·3
	12. ii. 31	537	10·6	6	26·9	253·5	111·9	138·9
	1. iv. 31	532	6·0	9	22·7	375·7	108·2	130·9
20	18. xii. 30	396	7·9	4	16·1	202·6	104·4	120·5
	12. ii. 31	433	10·5	5	20·0	189·9	111·1	131·1
	1. iv. 31	435	9·3	5	22·2	237·8	91·1	113·3
	14. v. 31	454	6·2	8	24·9	401·6	116·8	141·7
21	15. i. 31	310	7·6	2	21·2	280·4	96·3	107·5
	19. iii. 31	358	9·6	7	17·2	178·6	90·7	107·9
	30. iv. 31	383	2·7	7	16·0	592·6	80·9	96·9
	14. v. 31	375	2·4	9	12·7	529·1	98·4	111·1
23	19. ii. 31	374	8·1	8	15·0	185·2	63·5	78·6
	23. iv. 31	444	5·0	8	17·2	344·0	105·4	122·6
	18. vi. 31	488	4·5	8	18·2	404·5	81·8	100·0
	16. vii. 31	481	6·0	11	19·5	325·0	75·5	95·0
26	19. ii. 31	322	12·0	5	16·6	138·3	71·7	88·3
	30. iv. 31	347	11·3	4	15·7	138·9	77·2	92·9
	21. x. 31	396	11·1	5	20·3	182·9	81·6	101·9
	6. i. 32	416	5·4	7	15·6	288·8	88·9	104·5
	1. iii. 32	394	10·3	6	19·2	186·5	103·9	123·1
27	12. iii. 31	271	10·2	5	20·6	202·0	93·0	113·6
	7. v. 31	321	10·6	4	22·2	209·5	68·3	90·5
	24. vi. 31	339	6·0	7	22·1	368·3	97·7	119·8
	17. viii. 31	353	8·3	9	20·4	245·7	89·8	110·1
	30. ix. 31	358	5·3	9	16·5	311·3	100·8	114·5
	6. i. 32	388	0·8	8	16·2	2025·0	82·1	98·3
29	21. iv. 31	286	5·7	5	16·9	296·5	90·0	107·0
	16. vi. 31	329	3·9	10	11·9	305·1	92·9	104·8
	28. vii. 31	349	9·3	5	21·9	235·4	93·6	115·5
	29. ix. 31	386	1·8	2	13·3	739·0	99·3	112·6
31	13. viii. 31	357	8·7	9	21·6	248·3	86·9	108·5
	30. ix. 31	376	9·5	5	17·8	187·4	98·0	115·8
	6. i. 32	403	4·8	9	19·8	412·6	99·5	119·3
	2. iii. 32	388	8·2	7	22·5	274·5	91·9	114·3
	4. v. 32	406	9·7	6	16·9	174·3	79·9	96·8
	27. vii. 32	425	11·3	2	18·3	162·0	78·0	96·3
32	30. vii. 31	385	11·1	6	22·3	200·9	106·9	129·2
	28. ix. 31	356	12·3	5	18·5	150·4	75·4	93·9
	6. i. 32	401	5·6	5	15·6	278·6	78·3	93·9
	1. iii. 32	392	7·3	8	18·4	252·1	98·2	116·6
	4. v. 32	398	10·5	5	23·4	222·8	70·9	94·3
	8. vi. 32	409	8·2	6	18·4	224·4	86·4	104·8
	22. vi. 32	409	9·7	5	16·9	174·2	84·2	101·1
	6. vii. 32	396	8·0	6	14·7	183·7	80·8	95·5
35	11. xi. 31	243	5·2	7	15·1	290·4	66·0	81·1
	13. i. 32	291	5·2	5	11·2	215·4	79·3	90·5
	9. iii. 32	310	5·9	6	12·7	215·2	84·3	97·0
	11. v. 32	334	6·9	5	15·0	217·4	83·1	98·1
	13. vii. 32	381	9·1	4	18·5	203·4	79·7	98·2
37	11. xi. 31	294	8·8	5	18·9	214·8	81·9	100·8
	13. i. 32	339	7·2	5	17·5	243·1	107·8	125·3
	8. iii. 32	340	4·7	6	16·4	348·9	108·3	124·7
	11. v. 32	355	5·2	6	13·0	250·0	94·8	107·8
	8. vi. 32	374	6·9	5	18·8	272·5	88·3	107·1
	22. vi. 32	369	11·2	3	17·7	158·0	82·6	100·3
	6. vii. 32	368	8·7	7	14·0	161·0	77·1	91·1

Calculation of results.

The formula used to calculate the biological values of proteins

$$\left(100 \times \frac{\text{Body nitrogen saved}}{\text{Food nitrogen absorbed}}\right)$$

from the determinations of the nitrogenous exchange is derived from the graph given by Martin and Robison [1922], *viz.*

Biological value of protein $X = 100 \times (1 - \tan \theta)$, $\tan \theta$ being equivalent to

$$\frac{\text{Total N output in exp. with } X - \text{total N output on nitrogen-free diet}}{\text{True intake of N in exp. with } X}.$$

The nitrogen ingested during the nitrogen-free experiment is so small in amount, compared with the other values involved, that it can be disregarded.

The chief difficulties which arise in connection with obtaining a trustworthy estimate of the biological value of a protein by this method are the following.

(*a*) *The determination of the amount of faecal nitrogen of endogenous origin.* This figure is required for calculating all three terms in the above expression. In the first place, the total N output in the experiment with X is made up of the urinary nitrogen *plus* the endogenous faecal nitrogen. Secondly, the endogenous faecal nitrogen forms part of the total nitrogen output on the nitrogen-free diet. Thirdly, it is needed in order to calculate the amount of unabsorbed food nitrogen in the experiment with X (total faecal nitrogen − endogenous faecal nitrogen), which must be subtracted from the total nitrogen intake to get the true intake.

(*b*) *The proper interpretation of the variations occurring in the amount of total output of endogenous nitrogen*, at different times, with the same rat when receiving a nitrogen-free diet.

Determination of endogenous faecal nitrogen.

Mitchell [1924] argued that the amount of faecal nitrogen of endogenous origin excreted by a given animal was proportional to the weight of dry food ingested and that this ratio varied for different rats. In our experience however, the results of several series of experiments on the same rat receiving nitrogen-free diets (see Table I, col. 7) provide no evidence in support of such a relationship, at any rate where adult rats are concerned. On the contrary, in our work, the same rat has shown an excretion of faecal nitrogen which is roughly constant in amount, in spite of large variations in the food intake. Of 57 determinations made on 11 rats, 44 showed the endogenous faecal nitrogen excreted to be between 16 and 23 mg. daily. It was, decided, therefore, to take the actual amount of nitrogen excreted in the faeces of each rat during the period of nitrogen-free feeding as the measure of that particular animal's faecal output of endogenous nitrogen. Neither by this method nor by that used by Mitchell is it possible to make any allowance for the problematical influence on this amount of increased roughage or of a higher proportion of nitrogen in the diet. In any case, the value of the faecal nitrogen is so much smaller in amount than the other figures involved in the equation that

only very large variations in this figure would exert a significant influence on the final results.

Variation in total basal nitrogen expenditure.

In Mitchell's experiments young growing rats were used and the amount of nitrogen, per 100 g. body wt., excreted in the urine on a nitrogen-free diet was taken to be the measure of each animal's basal nitrogen metabolism, that excreted in the faeces being disregarded. In the present series in which the rats were full-grown, the quantity of nitrogen excreted in the urine was not found to be directly related to the body weight. Changes in the latter, as pointed out above [Boas Fixsen, 1930], usually indicated the deposition or removal of fat rather than alterations in the amount of active tissue. In some instances the endogenous urinary nitrogen remained stationary or became less as the rat became heavier (see rats 23, 31, 37, 35, Table I). Generally, the amount remained roughly constant and, of the 57 determinations given in the 8th column of Table I, in 47 the daily urinary nitrogen excretion lay between 78 and 120 mg. although the variations in body weight ranged from 271 to 537 g. As regards the range of variation in basal nitrogen expenditure shown in determinations made on the same rat at intervals of 6 to 8 weeks, Mitchell considered, on the basis of determinations carried out at shorter intervals, that these variations would be gradual and steady both in amount and direction, and that, therefore, the basal nitrogen expenditure at any period could be read off from curves in which the results of the different estimations had been plotted against time.

In the present investigation it was decided to take, as the estimate of daily nitrogenous expenditure for any particular rat, the average of all the determinations of total nitrogen output on a nitrogen-free diet which had been made on that animal within a period of 3 months before and 3 months after the experiment under consideration.

Summary.

Certain modifications are described in the method used to estimate the biological values of proteins from the results of measurements of the nitrogenous exchange. These are concerned with securing an adequate and uniform intake of calories and with obtaining a satisfactory estimate of the basal nitrogen metabolism.

We wish to express our gratitude to Dr H. Chick for her constant help and advice throughout this investigation. One of us (M. A. B. F.) is indebted to the Medical Research Council for a part time grant and to the Lister Institute for hospitality.

REFERENCES.

Boas Fixsen (1930). *Biochem. J.* **24**, 1794.
Chick and Roscoe (1930). *Biochem. J.* **24**, 1780.
Martin and Robison (1922). *Biochem. J.* **16**, 407.
Mitchell (1924). *J. Biol. Chem.* **58**, 873.

CCXXIX. THE BIOLOGICAL VALUES OF PROTEINS.

IV. THE BIOLOGICAL VALUES OF THE PROTEINS OF WHEAT, MAIZE AND MILK.

By MARGARET AVERIL BOAS FIXSEN
and HESTER MARY JACKSON.

From the Division of Nutrition, Lister Institute, London.

(*Received October 12th, 1932.*)

The relative efficiencies of the proteins of wheat, maize and milk in supplying the amino-acid requirements of the body have gained particular interest in connection with current theories of the etiology of pellagra [Wilson, 1921; 1930], a disease now generally accepted to be of dietetic origin. The majority of recorded outbreaks of pellagra have occurred among the poorer classes in districts where maize and maize products form the staple foodstuffs of the population and its occurrence among populations subsisting on wheat and rice, though recorded, is extremely rare. The disease, if not too far advanced, is cured after change to a "good diet" in hospital or elsewhere, and milk, meat and yeast have been shown to possess preventive and curative powers when added to a pellagra-producing diet [Goldberger, Waring and Tanner, 1923; Goldberger and Tanner, 1924; Tscherkes, 1930]. It is therefore reasonable to expect that any hypothesis as to the cause of the disease should account satisfactorily for these facts [see Aykroyd, 1930, 1].

Goldberger's vitamin theory [Goldberger and Tanner, 1925; Goldberger, Wheeler, Lillie and Rogers, 1926; Goldberger, 1927] attributes pellagra to a deficiency of the heat-stable vitamin B_2 component of the yeast vitamin B complex and would explain the curative action of the foodstuffs mentioned above on the ground of their high content of this vitamin. It does not, however, account for the association of pellagra with the consumption of maize, for Aykroyd and Roscoe [1929] have demonstrated that maize contains appreciable quantities of vitamin B_2, being in this respect in no wise inferior to wheat.

Turning to the theory elaborated by Wilson [1921; 1930] that pellagra is due to an amino-acid deficiency, *i.e.* to the ingestion of diets containing proteins of low biological value, the evidence is somewhat conflicting. Our present information as to the biological values of the mixed proteins of foodstuffs is derived from experimental work in which the three following methods have been employed.

(*a*) McCollum and Simmonds [1917], following a method used by Osborne and Mendel [1916] in their work on pure proteins, fed young rats on diets in which the foodstuff under investigation was the sole source of protein and determined the minimum percentage of protein in this form which must be given in the diet in order to prevent loss of weight. Using this method they concluded that the proteins of whole wheat and whole maize were of approximately equal nutritional value.

(*b*) In later work [McCollum, Simmonds and Parsons, 1921] the diets containing the foodstuffs to be investigated were so constituted that in each the proportion of protein was the same. The performances of rats fed on the different diets were then compared, not only as regards rate of growth, but also as regards longevity, reproduction and lactation, during, if possible, at least two generations. On the basis of experiments of this nature they were able to range the proteins investigated in the following descending order of merit as regards their biological value:

$$\text{Ox kidney} > \text{wheat} > \begin{Bmatrix}\text{milk}\\ \\ \text{ox liver}\end{Bmatrix} > \begin{Bmatrix}\text{ox muscle}\\ \text{barley}\\ \text{rye}\end{Bmatrix} > \begin{Bmatrix}\text{maize}\\ \\ \text{oats}\end{Bmatrix} > \begin{Bmatrix}\text{soya beans}\\ \text{navy beans}\\ \text{peas}\end{Bmatrix}$$

In this series the high value assigned to wheat and the low position of maize are noteworthy, and afford support to Wilson's theory of the aetiology of pellagra. Since, however, in these experiments the foodstuff tested was frequently the sole source not only of the protein, but also of the vitamin B complex, variations in the supply of one or other of the constituents of this complex might have influenced the results.

(*c*) The third method involves experiments of comparatively short duration and consists in determining the relative efficiency of different proteins for replacing the daily nitrogenous expenditure of the body—the "wear and tear" nitrogen—and for supplying the material for growth. The determination is made by the balance sheet method elaborated by Thomas [1909; 1910]. The figures obtained by Thomas in a heroic series of experiments on himself assigned the lowest value to the proteins of maize. The relative quantities of body nitrogen which could be replaced by 100 parts of the protein-nitrogen supplied from the following foodstuffs were as follows: from meat 109; milk 100; white wheat flour 40; maize 30. Thomas's figures were used by Wilson [1921] in his development of the theory that pellagra is the result of an amino-acid deficiency. It is obvious that calculations made on this basis would show diets containing a large proportion of maize to be markedly inferior in the biological value of their total proteins to those consisting largely of wheat, even if other deficiencies were present in the latter diets. Thomas's technique and conclusions have, however, been severely criticised from many points of view by Martin and Robison [1922].

McCollum [1911; 1914] also used the balance sheet method, working with pigs, and obtained results which, calculated by Thomas's formula [see

Chick, 1930] yielded the following estimates of biological value; milk, 74; caseinogen, 67; maize, 51; wheat, 45. A further series of experiments on the pig [Hart and McCollum, 1914] also showed the biological values of the proteins of wheat, maize and oats to be about equal. In these experiments the diets contained high percentages of protein.

The results obtained by Mitchell and his co-workers [Mitchell, 1924, 1, 2; Mitchell and Carman, 1924; 1926; Mitchell and Beadles, 1926] with young growing rats are also of interest. When the diet contained 8 to 10 % of protein, they obtained the following estimates of the biological value of the proteins of certain foodstuffs: whole milk, 85; liver or kidney, 77; ox muscle, 69; wheat, 67; maize, 60; white flour, 52. In these experiments the vitamin B complex was supplied as Harris yeast vitamin.

From the above facts it is apparent that the available data as to the relative nutritional values of the proteins of the different foodstuffs are extremely conflicting, whether the criterion employed in the tests has been the value for maintaining nitrogenous equilibrium in short-period tests or the value for supporting growth and development in long-period trials. The whole subject is reviewed in great detail in a critical article by Mitchell [1924, 3], to which reference should be made.

On the whole there is evidence for the superiority in nutritive value of animal protein over cereal protein, though McCollum and his co-workers found wheat proteins better than those of milk or ox muscle. They also found wheat superior to the other cereals tested including maize and in this advantage of wheat over maize they confirmed the result obtained by Thomas. But in the other researches cited, no significant difference has been demonstrated between the values of the two cereals.

The contradictory nature of these results suggested that a fresh assay of the biological values of some of the more representative foodstuffs, more especially wheat and maize, would be of interest, particularly as it is now possible to supply the constituents of the vitamin B complex in concentrates of relative purity, thus obviating some of the difficulties inherent in many of the estimations carried out previously [see Boas Fixsen, 1930].

Experimental.

Method.

The balance sheet method was used and the technique employed was that fully described in previous papers [Chick and Roscoe, 1930; Boas Fixsen, 1930] with the modifications given in the preceding paper [Boas Fixsen and Jackson, 1932].

Materials used.

Wheat products: whole wheat, white flour and wheat germ prepared from the same sample of soft red English wheat.

Maize products: whole maize and maize endosperm from the same sample of South American yellow corn.

Cow's milk: a preparation of whole winter milk dried by the roller process.

Caseinogen: "light white casein" (British Drug Houses), a sodium caseinogenate, manufactured by the method of Hammarsten.

Heated caseinogen: "light white casein" heated for 3 days at 112°, being stirred at regular intervals.

Lactalbumin: a sample prepared from whey.

"*Whole rat.*" After removal of the skin, six adult rat carcases were placed in boiling water for 10 minutes. The lungs, livers, hearts, kidneys and muscular tissue were minced, dried at 37° for 36 hours and finely ground. 1 g. of the product was the equivalent of 3·2 g. of the fresh material and contained 10·37 % of nitrogen.

Diets.

The nitrogen-free diet used had the following composition.

Corn starch	735 g.
Sugar	90
Clarified beef dripping	100
Cod-liver oil	20
Salt mixture	50
Calcium carbonate	8

The nitrogen content of different batches varied from 0·2 to 0·4 % of the dry weight and the calculated calorie value of the dry diet was 460 kg.-cal. per 100 g.[1]

In the experiments with "whole rat" these materials were administered by the method described in a previous communication in connection with heated caseinogen [Boas Fixsen, 1930]. The other substances tested were incorporated in diets so constituted that the ratio of fat to carbohydrate remained approximately the same. Table I gives the composition of two

Table I. *The constitution of two representative diets—WG and WF—in which the protein was derived from wheat germ and white flour respectively.*

The weights given refer to the air-dry materials.

		Diet WG	Diet WF
		g.	g.
Wheat germ*, 220 g.	Protein	88	—
	Fat	26	—
	Carbohydrate	54	—
	Undetermined	52	—
White flour*, 600 g.	Protein	—	74
	Fat	—	8
	Carbohydrate	—	507
	Undetermined	—	11
Corn starch		600	226
Sugar		90	90
Clarified beef dripping		104	124
Cod-liver oil		20	20
Salt mixture		50	50
Calcium carbonate		8	8

* The figures for composition are taken from Osborne and Mendel [1919].

[1] The calorie value of this diet was previously wrongly given as 380 kg.-cal. per 100 g. [Boas Fixsen, 1930].

representative diets in which the protein was provided by wheat germ and wheat endosperm respectively, and Table II shows the estimated nitrogen and water contents and the calculated calorie values of the fat and carbohydrate of each of the diets used.

Table II. *Details of the diets used.*

Figures for water and nitrogen obtained by direct analysis.

Source of protein	Water content %	Nitrogen content of dry diet %	Protein content of dry diet (N × 6·25) %	Non-protein kg.-cal. per g. of dry diet
Whole wheat	10·10	0·890	5·6	4·1
White flour	10·32	1·125	7·0	4·2
Wheat germ	10·38	1·093	6·8	4·0
Whole maize	12·34	1·283	8·0	4·1
,,	8·00	0·781	4·9	4·4
Maize endosperm	9·50	1·072	6·7	4·6
Whole milk	8·66	1·097	6·9	4·4
Caseinogen	9·68	0·919	5·7	4·4
Heated caseinogen	9·02	1·110	6·9	4·5
Lactalbumin	9·54	1·074	6·7	4·5

Influence on biological value of the proportion of protein in the diet. Thomas [1909; 1910] assumed that, if sufficient fat and carbohydrate were supplied to cover the energy requirements of the organism, the capacity of any protein to replace the nitrogenous waste of the body tissue would remain constant at all levels of intake. Martin and Robison [1922] questioned the validity of this assumption on general grounds but found, nevertheless, in their own experiments made with the proteins of whole wheat, that the figure obtained for biological value remained the same when the diet contained the protein in widely different proportions. One of the present authors found the same to be true of heated purified caseinogen [Boas Fixsen, 1930]. Mitchell and other workers [Mitchell, 1924, 1; Morgan, 1931] on the other hand, found the biological values of many proteins to decline as the proportion in the diet was increased. In all the above tests the balance sheet method was used.

In the case of the proteins forming the subject of the present investigations, all those that have as yet been studied in different proportions in the diets have been found to show an increased biological value when given at the lower levels. This point is being made the subject of further study.

When the present investigation was planned it was not anticipated that the estimates of biological value would be influenced by the proportion of the protein in the diet. For this reason this level was not made uniform for all the proteins used. Whole wheat and caseinogen were studied in experiments so arranged that the proteins formed 6 % of the diet, with whole maize the levels of protein intake were 8 and 5 % and, with the other substances tested, 7 %. This has to be taken into account when deducing relative biological values from the results obtained.

Results.

The results of the experimental work are collected in Table III. The average biological values, given in the last column, agree fairly well with those of Mitchell.

Table III. *Details of the experiments in which the biological values of proteins in various foodstuffs were determined.*

The figures represent average daily values.

Source of protein used	% of protein in diet	Date of exp.	Rat no.	Body weight g.	Change in body weight g.	Intake of non-protein Calories per kg. body weight kg.-cal.	Intake of nitrogen (true) mg.	Output of nitrogen: Urine mg.	Output of nitrogen: Endogenous faecal mg.	Output of nitrogen: Total mg.	Biological value	Average
Whole wheat	6	29. iii.–2. iv. 31	21	379	+1	174	122·4	117·1	16·8	133·9	77	
		13–17. iv. 31	20	460	−3	124	98·6	138·9	20·8	159·7	67	
		,,	21	399	−2	138	98·6	121·7	16·8	138·5	67	
		27. vi.–1. vii. 31	23	503	−2	144	133·7	141·7	18·4	160·1	59	68
Wheat germ	7	16–20. xi. 31	26	420	−2	129	127·0	126·8	18·0	144·8	67	
		,,	31	406	−1	142	134·2	133·8	19·7	153·5	71	
		,,	32	416	−1	138	129·9	140·2	17·1	157·3	51	
		,,	27	408	−1	142	130·5	126·4	17·8	144·2	75	
		,,	34	401	−1	155	136·7	130·7	19·4	150·1	83	69
Wheat endosperm	7	11–15. v. 31	26	362	−3	136	123·6	123·7	16·2	139·9	60	
		27. iv.–1. v. 31	20	460	−2	117	129·9	159·3	19·4	178·7	56	
		4–8. v. 31	23	454	+1	139	151·0	145·7	17·5	163·2	57	
		20–24. iv. 31	27	321	+2	192	155·9	133·1	21·8	154·9	69	
		,,	28	328	−1	154	123·1	120·5	17·6	138·1	62	61
Whole maize	8	17–24. iii. 31	12, 15	393	+1	185	195·6	147·0	22·9	169·9	63	
		,,	11, 16	383	+2	188	177·5	138·6	15·7	154·3	71	
		17–21. iii. 31	13, 14	375	+1	142	144·9	108·9	32·9	141·8	68	67
	5	26. iv.–1. v. 31	11, 15	418	−2	139	79·6	97·0	19·5	116·5	85	
		,,	12, 16	416	−2	160	99·5	96·2	20·3	116·5	78	
		,,	13, 14	392	−1	151	97·3	92·2	22·9	115·1	81	
		29. xii. 30–2. i. 31	20	414	−3	119	76·5	114·1	18·1	132·2	92	84
Maize endosperm	7	9–13. ii. 31	21	371	−2	187	135·0	134·9	16·8	151·7	66	
		,,	22	389	−4	173	135·4	136·0	19·5	155·5	71	
		16–20. iii. 31	18	546	−2	133	150·8	142·6	23·2	165·8	77	
		,,	20	441	−3	123	122·4	149·3	20·8	170·1	65	70
Whole milk	7	20–24. iii. 32	37	342	+1	136	103·4	111·3	15·6	126·9	93	
		,,	41	305	−1	172	119·1	84·3	26·1	110·4	88	
		,,	35, 36	345	−1	129	89·3	104·6	13·3	117·9	77	
		4–8. iv. 32	31	408	−1	136	118·3	105·7	19·7	125·4	87	86
Lactal-bumin	7	1–5. ii. 32	42	308	−4	126	83·1	108·6	11·3	119·9	70	
		,,	26, 31	406	−3	131	121·8	137·3	19·2	156·5	66	
		,,	32	416	−2	139	128·7	135·7	17·0	152·7	63	
		8–12. ii. 32	36	345	−4	147	106·6	130·6	14·4	145·0	60	65
Caseinogen	6	23–27. xi. 31	35	262	+2	169	94·0	78·0	13·2	91·2	94	
		,,	37	403	−1	144	118·0	124·2	18·3	142·5	75	
		,,	39	388	−3	116	87·9	95·6	19·9	115·5	79	
		10–14. i. 32	38	337	−1	133	91·9	100·1	7·5	107·6	81	
		25–29. i. 32	39	316	−6	157	106·9	125·4	18·2	143·6	57	
		,,	37	344	−1	156	114·1	127·5	17·6	145·1	75	
		,,	35	300	−1	159	94·4	101·6	13·0	114·6	74	76
Heated caseinogen	7	22–26. ii. 32	41	299	+1	167	115·6	119·3	26·1	145·4	57	
		,,	43	361	−4	146	121·2	169·0	18·4	187·4	49	
		28. ii.–3. iii. 32	42	297	−2	180	117·9	135·0	11·3	146·3	57	54
"Whole rat"	7	27. vi.–1. vii. 32	31	431	−1	>122	139·7	157·3	17·6	174·9	44	
		,,	35	377	0	>142	143·0	132·9	16·8	149·7	64	
		,,	41	355	−1	>145	134·9	135·4	22·8	158·2	53	
		,,	43	424	−1	>130	140·0	149·0	19·5	168·5	57	
		25–29. vii. 32	41	361	−1	>141	145·1	137·2	20·7	157·9	57	55

Seeing that the figures obtained from different experiments with the same protein showed variations of from 10 to 20 %, only large differences between the average figures obtained for the different proteins are considered to be of significance. When this is allowed for, the following conclusions can be drawn.

Wheat and maize proteins. There is little significant difference between the values found for whole maize (67 and 84 at 8 and 5 % levels, respectively) and whole wheat (68 at a 6 % level). Wheat germ gave a value of 69 (7 % level) and wheat endosperm 61 (7 % level) which is in accord with the observation of Klein *et al.* [1926] that in wheat the proteins of the germ are superior to those of the endosperm.

Milk protein. The proteins of whole milk were found to possess the highest value of the series tested, with an average figure of 86 when given in a diet containing 7 % protein. Caseinogen and lactalbumin were both inferior, giving figures of 75 and 65 for protein intakes of 6 % and 7 % respectively, suggesting that these two proteins exert a supplementary action upon one another in their mixture in milk. The slight superiority of caseinogen over lactalbumin is contrary to what would be expected from the available data concerning the amino-acid make-up of these two proteins, seeing that analysis of the latter shows a larger proportion of the important amino-acids tryptophan, cystine and lysine. Osborne and Mendel [1916] found lactalbumin to be superior to caseinogen for both growth and maintenance of young rats.

Purified caseinogen (extracted with acidified water and with dilute alcohol and then heated for 3 days at 115°) when evaluated previously [Boas Fixsen, 1930], was found to have an average biological value of 45 (50 when calculated by the modified method now used) at all levels. The discrepancy between this figure and the higher values obtained by other workers [McCollum, 1914; Mitchell, 1924, 2] was at that time tentatively ascribed either to differences in technique, or to the removal during purification of traces of a contaminating protein with a biological value higher than that of caseinogen. Morgan's work [1931] has shown, however, that it should be attributed to the marked deterioration in biological value suffered by a protein as the result of prolonged heating at a relatively high temperature. This is confirmed in the present work, in which unpurified caseinogen was evaluated at 76 (6 % level), while caseinogen which had been heated but not extracted had sunk in value to 54 (7 % level).

The low value of 55 given by the "whole rat" preparation is of interest. Theoretically one would expect the proteins of the rat to have for that animal a value approaching 100, by analogy with Michaud's [1909] experiments in which dog's flesh was found to have an efficiency of 100 % in replacing the dog's daily nitrogen excretion.

Applicability of the experimental results to the current theories of the aetiology of pellagra.

It is difficult to reconcile the above observations on wheat and maize with the theory that pellagra is caused by an inferiority in the quality of the protein

consumed on the maize diets, *i.e.* to an amino-acid deficiency. Since 58 % of the protein of maize [Osborne and Mendel, 1913] is the notoriously incomplete zein, one must conclude that maize glutelin, which constitutes 36 % of the total protein, forms an effective supplement to zein in maintaining nitrogenous equilibrium in the adult animal. Osborne and Mendel [1914], indeed, found maize glutelin to possess a high nutritive value.

On the other hand it is not easy to decide how far results of the kind described in this paper obtained in short term experiments on rats are applicable to the case of human beings subsisting for long periods on a diet consisting largely of maize or to what extent they are of importance in the aetiology of pellagra, a human disease developing after many months' subsistence on a faulty dietary regime.

It is worth noting, nevertheless, that six days (the duration of each metabolism experiment including the preliminary period) in a rat's life is roughly equivalent to 4 to 5 months of a man's life. In Goldberger's Rankin farm experiment [Goldberger and Wheeler, 1920] the first symptoms of pellagra appeared after 5 months' subsistence on the experimental diet.

The balance sheet method as used by us is concerned only with the relative efficiency of proteins in replacing the daily nitrogenous expenditure of the body, *i.e.* with the function of maintenance. In McCollum's long-term experiments, on the other hand, growth, reproduction and lactation were also involved. It is probable that for these functions a disparity exists between proteins shown to be of equal value as regards maintenance[1]. The inferiority of maize to wheat, when given as sole source of protein in diets employed for rearing young rats to maturity, has received some measure of confirmation in recent experiments in this laboratory. Pellagra, however, as was pointed out by Mitchell [1924, 3] in this connection, is a disease mainly of adult life and not specifically associated with periods of growth or adolescence.

In considering the theory linking pellagra with a simple amino-acid deficiency, a fact to be noted is the low calorie value of all the pellagrous diets quoted by Wilson, except that used in Goldberger's Rankin farm experiment. In consequence, much of the already low protein intake must have been used for fuel, so that whatever the biological value of the protein when used solely to supply nitrogen, a deficiency of protein would inevitably result. In every case cited where pellagra was cured by the addition to the diet of animal protein, the calorie value was at the same time raised, thus automatically improving the value of what was previously the sole source of protein. Many of the non-pellagrous diets were also of low calorie value, so that in these also the biological value of the protein must have been much lower than that calculated by Wilson, even if Thomas's figures from which the calculations were made are considered trustworthy.

[1] It is interesting to note in this connection that in South Carolina mill villages in 1917 the incidence of pellagra per 1000 persons from 20–29 years of age was 61 females to 3 males, while the ratio of married pellagrins to those found among single women was 52 to 12 [Smith, 1931].

It is evident, therefore, that the experimental support for the theory connecting pellagra with a simple amino-acid deficiency in the diet, is not as strong as has been supposed. The superiority in biological value shown by proteins of animal origin is in accord with the proved curative value of animal foods, but the fact that maize has not been found consistently inferior in this respect to wheat or certain other cereals fails to account for the known association of the disease with the consumption of maize. In this respect, therefore, the theory relating pellagra directly to a deficiency of protein of high biological value has not been more fortunate in its experimental support than the theory of vitamin deficiency put forward by Goldberger and his associates (see above).

It must be admitted therefore that as yet there is no satisfactory explanation of the fact, well established in human experience, that maize possesses a nutritive value inferior to that of wheat and that there is a greater liability to nutritional disease on diets consisting largely of the former cereal. Maize is eaten of necessity and never of choice if wheat is available, which fact probably provides an additional proof of its inferiority. The theory has frequently been advanced that a toxic substance is present in certain portions of the maize grain or may be developed under certain conditions. There is, however, very little direct evidence in its support. The oft-quoted experimental work of Horbaczewski [1910; 1912] was interpreted to show that the skin lesions of pellagra were due to an increased sensitiveness to strong sunlight caused by absorption of this toxin. Although his experiments lack confirmation [Aykroyd, 1930, 2] the widespread suspicion that some definitely unwholesome substance may be present in maize deserves further investigation.

Summary.

1. The following figures have been obtained for the biological values of the proteins of certain foodstuffs, using the balance sheet method with adult rats as the subjects, biological value being defined as the number of parts of body nitrogen replaced by 100 parts of the protein investigated,

$$\text{or} = 100 \times \frac{\text{Body N saved}}{\text{Food N absorbed}}.$$

Whole wheat (6 % protein in diet) 68; wheat embryo (7 %) 69; wheat endosperm (7 %) 61.

Whole yellow maize (8 %) 67; (5 %) 84; yellow maize endosperm (7 %) 70.

Whole milk (7 %) 86; lactalbumin (7 %) 65; caseinogen (6 %) 76; heated caseinogen (7 %) 54·5.

"Whole rat" preparation (7 %) 55.

2. From these figures certain conclusions have been drawn.

(*a*) No significant difference is shown between the biological values, as defined above, of the proteins of wheat and maize.

(*b*) The proteins of the embryo of wheat are of slightly superior value to those of the endosperm.

(*c*) Caseinogen suffers a marked decline in biological value as the result of prolonged heating at 112°.

(*d*) Caseinogen is a slightly more valuable protein for maintenance than is lactalbumin.

(*e*) Caseinogen and lactalbumin appear to have a supplementary action towards one another, the proteins of whole milk being superior in value to either protein ingested separately.

3. The experimental study of the biological value of maize and wheat proteins lends little support to the theory that pellagra among maize eaters is the result of a simple amino-acid deficiency. So far no satisfactory explanation is forthcoming of the general experience of mankind that diets consisting largely of maize have been found of inferior nutritive value to these containing wheat as the principal foodstuff.

We wish to express our gratitude to Dr H. Chick for her constant advice and criticism throughout the investigation. One of us (M. A. B. F.) is indebted to the Medical Research Council for a part time grant and to the Lister Institute for hospitality. We would also like to acknowledge the kindness of Messrs J. and H. Robinson Ltd., Flour Millers of Deptford, for supplying us with the samples of whole wheat, wheat germ and white flour used.

REFERENCES.

Aykroyd (1930, 1). *Brit. Med. J.* **i**, 647.
—— (1930, 2). *Biochem. J.* **24**, 1479.
—— and Roscoe (1929). *Biochem. J.* **23**, 483.
Boas Fixsen (1930). *Biochem. J.* **24**, 1794.
—— and Jackson (1932). *Biochem. J.* **26**, 1919.
Chick (1930). Recent advances in analytical chemistry, **1**, 166.
—— and Roscoe (1930). *Biochem. J.* **24**, 1780.
Goldberger (1927). *U.S. Pub. Health Rep.* **42**, 2193.
—— and Tanner (1924). *U.S. Pub. Health Rep.* **39**, 87.
—— —— (1925). *U.S. Pub. Health Rep.* **40**, 54.
—— Waring and Tanner (1923). *U.S. Pub. Health Rep.* **38**, 2361.
—— and Wheeler (1920). *Arch. Int. Med.* **25**, 451.
—— —— Lillie and Rogers (1926). *U.S. Pub. Health Rep.* **41**, 297.
Hart and McCollum (1914). *J. Biol. Chem.* **19**, 373.
Horbaczewski (1910). *Österr. Sanitätswesen*, 31.
—— (1912). *Österr. Sanitätswesen*, 21.
Klein, Harrow, Pine and Funk (1926). *Amer. J. Physiol.* **76**, 237.
McCollum (1911). *Amer. J. Physiol.* **29**, 210.
—— (1914). *J. Biol. Chem.* **19**, 323.
—— and Simmonds (1917). *J. Biol. Chem.* **32**, 347.
—— —— and Parsons (1921). *J. Biol. Chem.* **47**, 235.
Martin and Robison (1922). *Biochem. J.* **16**, 407.
Michaud (1909). *Z. physiol. Chem.* **59**, 405.
Mitchell (1924, 1). *J. Biol. Chem.* **58**, 905.
—— (1924, 2). *J. Biol. Chem.* **58**, 923.
—— (1924, 3). *Physiol. Rev.* **4**, 424.
—— and Beadles (1926). *J. Biol. Chem.* **71**, 429.
—— and Carman (1924). *J. Biol. Chem.* **60**, 613.

Mitchell and Carman (1926). *J. Biol. Chem.* **68**, 183.
Morgan (1931). *J. Biol. Chem.* **90**, 771.
Osborne and Mendel (1913). *Science*, **37**, 185.
—— —— (1914). *J. Biol. Chem.* **18**, 1.
—— —— (1916). *J. Biol. Chem.* **26**, 1.
—— —— (1919). *J. Biol. Chem.* **37**, 557.
Smith (1931). *Arch. Int. Med.* **48**, 907.
Thomas (1909). *Arch. Anat. Physiol.* 219.
—— (1910). *Arch. Anat. Physiol. Suppl.* 249.
Tscherkes (1930). Private communication.
Wilson (1921). *J. Hyg.* **20**, 1.
—— (1930). *Brit. Med. J.* i, 101.

CCXXX. THE CHEMISTRY OF MOULD TISSUE[1].
I. SOLUBLE CARBOHYDRATE CONSTITUENTS.

By ARTHUR GEOFFREY NORMAN, WILLIAM HAROLD PETERSON and RAY CLYDE HOUTZ.

From the Departments of Agricultural Chemistry and Agricultural Bacteriology of the University of Wisconsin, Madison, U.S.A.

(*Received October 15th, 1932.*)

No very detailed chemical examination of the carbohydrate constituents of any fungal material has ever been made, but numerous references exist to the isolation of individual polysaccharides from such tissue. In some cases the names which have been applied, *e.g.* fungal starch, cellulose, *etc.* are misleading, inasmuch as they suggest an identity with these plant constituents. The chemistry of fungal tissue has an individuality of its own and it is probably true to say that, while it differs in numerous respects from that of both plant and animal tissues, it more nearly approaches that of the latter than of the former. Protein and nitrogenous material are quantitatively more important than the carbohydrates in most cases, and frequently also the fat and combined fatty acid contents are quite high—conditions very different from those obtaining in mature plant tissues.

In the closing years of the last century much work was carried out on fungal mycelium from a histological point of view. Reliance was placed mainly on so-called selective staining reactions in combination with certain extraction procedures. Most of the workers concerned were impressed by the complex and resistant character of the tissue, and explained the difficulties they experienced in extraction on the basis of infiltration of one constituent into another and the presence of insoluble combinations between such constituents as fatty acids and proteins, cellulose and nitrogenous substances.

A number of carbohydrate preparations have been obtained from fungal material. Cramer [1894] isolated from the spores of *Penicillium glaucum* a polysaccharide giving a blue colour with iodine. This he termed spore starch, and showed that it could be completely hydrolysed in 3 hours by boiling with 1·25 % sulphuric acid. Mangin [1890] reported and studied a carbohydrate, callose, which he isolated from the hyphae of many species of fungi. He found it to be insoluble in water and alcohol, but easily soluble in cold alkali, and described a number of staining reactions for it, some of which are of questionable specificity. Winterstein [1894] obtained from *Boletus* by acid extrac-

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

tion a carbohydrate which he termed paradextran and later from *Polyporus* a similar material, para-isodextran. The latter he showed to yield glucose on hydrolysis, to give an intense blue colour with iodine, and to have $[\alpha]_D = +240°$ in solution in 5 % alkali. Since these preparations were apparently soluble in alkali, it is probable that they represent the same fraction as that obtained by Mangin and described as callose.

Dox and Neidig [1914, 1] prepared polysaccharides from the hot water extract of various fungi. From *Penicillium expansum* they obtained material, insoluble in cold water, termed by them mycodextran, highly dextrorotatory and yielding glucose only on hydrolysis. It was unattacked by enzymes and gave no coloration with iodine when pure. Later [1914, 2] they described a similar compound from *Aspergillus niger*, stated to give only galactose on hydrolysis. They express the opinion that both these polysaccharides are reserve substances to be used by the fungi when the carbohydrates in the medium are exhausted, since they were unable to obtain any from mature or partly autolysed cultures.

Boas [1917] investigated the cultural conditions necessary for the production of fungal "starch," but attempted no chemical work on this substance, relying on iodine coloration as an index of its presence. Schmidt [1925] considered the production of fungal "starch" in *Aspergillus niger* to be dependent on the nitrogen-carbohydrate ratio of the medium, a low ratio favouring "starch" production. The "starch," which was water-soluble, was hydrolysed by various enzymes and by acids. She believed it to be identical with amylose, and *iso*lichenin, but it must be pointed out that there is no quantitative evidence in support of this view. Takata [1929] isolated from *Aspergillus oryzae* a water-soluble preparation, said to be identical with that of Schmidt, and yielding glucose and a little mannose on hydrolysis either with acid or takadiastase. He also obtained an alkali-soluble polysaccharide by extraction with 10 % sodium hydroxide. This preparation had a nitrogen content of 4·4 % and was hydrolysed to reducing sugars only with difficulty. An appreciable amount of glucosamine was given on hydrolysis.

Raistrick and co-workers obtained a number of carbohydrates from fungi. Birkinshaw, Charles and Raistrick [1931] prepared from various species of *Penicillium digitatum* a hot water-soluble polysaccharide which separated out on cooling. This probably corresponds to the mycodextran of Dox and Neidig [1914, 1] since it yielded only glucose on hydrolysis, was highly dextrorotatory, gave no colour with iodine and was unaffected by diastatic enzymes. From *Fumago vagans*, Birkinshaw and Raistrick [1931] isolated a water-soluble carbohydrate, precipitable by alcohol, also yielding only glucose on hydrolysis and unaffected by enzymes.

Most of the investigations mentioned above deal with one isolated constituent of the mould tissue, and few attempts have been made to examine the tissue as a whole. Perhaps the only recent survey which has been made is due to Thomas [1928] working with the hyphae of *Fusarium*, and later [1930] also

with *Sclerotium*. Like the earlier workers he too relied extensively on staining reactions and colorimetric tests and few analytical data are given. He points out that the apparent resistance of the hyphae towards most reagents is due to the presence of fatty substances and fatty acids in the cell-wall and postulates cellulose-fatty acid, and protein-pectin complexes incrusting and impregnating a basic skeleton of a chitinous nature.

This review would seem to indicate that different species show considerable variation in the nature of the carbohydrate material present. The majority of these preparations are, however, small fractions easily soluble, and do not represent the larger structural components which may, as in the higher plants, exhibit more constancy.

Experimental.

(*a*) *Material employed.*

In the work to be described, the fungal tissue investigated was from one strain of *Aspergillus fischeri* only. It was obtained from Dr Charles Thom of the U.S. Department of Agriculture, and was known by him as No. 5041 of his collection. It develops on liquid media in the form of a thick mycelial felt or pad, and by cultivation in pans in a specially designed incubator of the type described by Raistrick *et al.* [1931] and Peterson *et al.* [1932] may be obtained in quantities sufficient for extensive chemical investigation. The medium throughout was glucose-ammonium nitrate[1], initially at a p_H of 6·8 and the conditions of fermentation were those outlined by Peterson *et al.*

When harvested, the mycelium was usually not very heavily sporulated, though considerable variation in this and in the mycelial weight from a given concentration of sugar was observed. In general the tissue taken was of the same age, but it was evident that it was not always of equal maturity. Since there is no rational criterion of maturity, it was not possible to examine the effects of such differences.

The tissue was available in four different conditions: (1) fresh, (2) killed by heating to 99°, (3) killed at 99° and dried at 67°, (4) killed at 99°, dried at 67° and extracted for many hours with alcohol at 80°. Of these, the fresh material is much to be preferred. The process of killing by heat followed by drying or treatment with alcohol seems to produce changes in physical properties both of the protein and carbohydrate constituents, which render subsequent handling more difficult and give preparations of a poor colour. The medium employed for the growth of the mould had a high content of inorganic salts and despite careful washing of the mycelial felt, salts (mainly phosphate, magnesium and sulphate ions) gave considerable trouble later in the preparations. The underside of the pad was of a firm gelatinous nature and appeared to retain these physically.

[1] The composition of the medium was: cerelose (glucose), 100 g.; NH_4NO_3, 10 g.; KH_2PO_4, 6·8 g.; $MgSO_4, 7H_2O$, 5·0 g.; *M*/1 $FeCl_3$, 0·5 cc.; 1 % $ZnSO_4, 6H_2O$ sol., 5·0 cc.; water, 1000 cc.

(*b*) *Distribution of constituents.*

A typical sample of dried tissue gave on analysis C, 43·8 %; N, 5·4 %; ash, 5·67 %. The nitrogen content varied with different samples, but was over 5 % in every case, the highest figure obtained being 6·02 %.

A preliminary survey of the distribution of constituents was made by determining the loss on treatment with various reagents. This indicated that the carbohydrate material of the tissue may be placed in three groups: (1) water-soluble, (2) alkali-soluble, (3) alkali-resistant residue. Protein was present in both watery and alkaline extracts along with the carbohydrate.

Typical figures are given in Table I for the watery and subsequent alkaline extracts of fresh living tissue (A), heat-killed and dried tissue (B) and heat-killed and alcohol-extracted tissue (C).

Table I.

Extract		% removed	N content of extract g.	N calc. as protein g.	Probable carbohydrate by difference %
Hot water	(A)	37·9	3·10	19·4	18·5
2 extractions	(B)	25·5	1·74	10·9	14·6
	(C)	12·8	0·59	3·7	9·1
Hot 2 % alkali	(A)	32·2	1·96	12·3	19·9*
3 extractions	(B)	36·2	3·14	19·6	16·6*
	(C)	43·2	4·30	26·9	16·3*

* Possible saponification of fats by this treatment has not been considered.

It will be seen that the amount of material extractable by these two processes was influenced to a marked degree by the condition of the tissue. The non-nitrogenous material, presumably carbohydrate, removable by water and alkali respectively was approximately equal in amount from the fresh and the dried tissue. In view of the higher percentage removal from fresh tissue, this was employed in practically all the preparations to be described.

(*c*) *Sugars present.*

About 2 g. of the dried tissue were hydrolysed by boiling for 5 hours with 3 % H_2SO_4. The filtrate was neutralised with barium carbonate, decolorised with norite and concentrated under reduced pressure to a small volume. On preparation of the osazone, glucosazone only was obtained. An attempt to obtain a hydrazone in the cold (mannose) was unsuccessful. The glucosazone was recrystallised from pyridine and the melting-point found to be identical with that of glucosazone (M.P. 204°).

A mucic acid test for galactose by oxidation of the tissue with nitric acid gave negative results. A uronic acid determination by distillation with 12 % HCl yielded only traces of CO_2 and indicated the absence of this group.

Furfuraldehyde estimations for pentoses were carried out on several samples and a trace only was found to be present. Care has to be exercised in carrying out this estimation on mould tissue since some fatty acid distils over with the HCl-furfuraldehyde and the distillate must be filtered before the

addition of phloroglucinol. The precipitated phloroglucide was extracted with hot alcohol in the usual manner to remove the phloroglucide of ω-hydroxymethylfurfuraldehyde due to hexose units.

Furfuraldehyde from alcohol-extracted tissue, 0·56 % = anhydropentose 0·96 %.

Furfuraldehyde from fresh tissue grown on 5 % xylose, 0·39 % = anhydropentose 0·67 %.

The nucleic acid of *Aspergillus oryzae* has been shown [Takata, 1929] to contain pentose, indeed pentose forms an appreciable percentage of the molecule. It would not be unreasonable to suppose therefore that this small quantity of pentose found in the tissue might be due to nucleic acid. Even with xylose as the sole source of carbon, the tissue appeared to contain only glucose units in its polysaccharides.

(d) Water-soluble carbohydrate.

(1) *Preparation and properties of the acid-alcohol precipitate* (Fraction A). When fresh material was used it was thoroughly washed with cold water, and ground to a pulp in a mill before extraction. Very satisfactory grinding was effected if the tissue was first frozen. When dried material was employed, this was ground to a fine powder and allowed to soak for several hours in order to get satisfactory penetration. Two extractions were then made with hot but not boiling water, and filtration was effected first on a cloth and then through paper. A golden yellow solution was obtained, filtering rapidly while hot, but only slowly when cold. No fraction comparable with the mycodextran of Dox and Neidig [1914, 1] separated out on cooling. The solution was concentrated under reduced pressure, again filtered and poured into 2·5 volumes of alcohol containing a little HCl. A white flocculent precipitate appeared, settling readily. The filtrate was retained. The precipitate was washed by repeated sedimentation in acid-alcohol and finally dried in alcohol of increasing concentration. It will be referred to as Fraction A. The importance of adequate washing cannot be overemphasised, for unless this was thorough, the finished preparation contained much ash. The quantity of the fraction precipitated by acid-alcohol seems to vary considerably with the material; in one or two cases practically none of this fraction was obtained. It dried easily to a white powder, soluble in water with some difficulty. Unlike the water-soluble fungal "starch" obtained by Schmidt [1925], by Boas [1917] and by Cramer [1894], it gave no coloration with iodine. Though the major part of the protein was no doubt coagulated by the heating process, this fraction usually contained a little nitrogen, presumably in the form of protein. The preparations were hydrolysed readily with dilute acid to give reducing sugar, characterised by means of its osazone as glucose. No hydrazone was obtained in the cold, indicating the absence of mannose. The mucic acid test for galactose and naphthoresorcinol test for uronic acids were both negative.

The dried preparations redissolved in water only with difficulty and gave

a somewhat dark solution. The rotation of one preparation was found to be $[\alpha]_D^{22°} = 86{\cdot}2°$.

When in solution this carbohydrate passed slowly through a cellophane membrane (Du Pont—600). The integrity of the membrane was subsequently verified by dialysing starch against a dilute iodine solution. The starch was retained.

The rates of hydrolysis of two typical preparations are given in Table II (sample I, ash 3·5 %; N, 1·33 %; sample II, ash 1·3 %; N, 0·87 %).

Table II.

% reducing sugar calc. as glucose.

Hours ...	½	1	1½	2	3	4	16	18	24
Sample I	18·2	46·5	58·3	59·5	64·7	67·4	—	—	80·3
Sample II	31·9	45·5	—	62·5	79·4	84·9	94·8	80·5	—

To obtain confirmatory evidence as to the nature of the sugar, rotations were determined after hydrolysis. 0·197 g. in 25 cc. 3 % H_2SO_4 was boiled for 5 hours, neutralised with barium hydroxide, boiled with a little norite, filtered and evaporated down to less than 5 cc. *in vacuo*. After making up to 5 cc. the rotation was observed in a 2 dm. tube as + 2·41°. The sugar concentration was then determined and found to be 0·0232 g. per cc. calculated as glucose. On this basis $[\alpha]_D = \frac{2{\cdot}41}{{\cdot}0232 \times 2} = + 51{\cdot}9°$. In view of the close approximation to the theoretical value for glucose, this observation provides a further indication that the main if not the only sugar to be obtained on hydrolysis of this carbohydrate is glucose.

(2) *Other water-soluble carbohydrate fractions.* An investigation of the alcoholic filtrate from the precipitation of the acid-alcohol fraction (Fraction A) showed that more carbohydrate material remained in solution and could not be precipitated by increasing the alcohol concentration. Two crude fractions were obtained, one by making the solution alkaline, and the other by the subsequent addition of lead acetate in acetic acid. The nitrogen content of these precipitates was low, and some glucose was obtained on hydrolysis. These fractions have not yet been further investigated.

(*e*) *Alkali-soluble carbohydrates.*

(1) *Preparation of crude material.* As shown earlier, an alkaline extract of mould tissue contains in addition to carbohydrate material considerable quantities of protein, possessing very similar properties in so far as precipitability is concerned. Preparations obtained by methods similar to those employed for the alkali-soluble constituents of plant material (hemicelluloses) have, in consequence, a high protein content and are of little value in work on the polysaccharides. The procedure generally followed in making an extract was to treat the residue first with 2 % NaOH in the cold, and then again with the same concentration of alkali slightly warmed. In some cases a treatment with hot 2 or 4 % NaOH followed, but in general this was omitted since the

object was not so much to obtain complete extraction of the tissue as to get a preparation of high purity. The extracts were obtained by filtration through muslin or cheese cloth and the residues pressed out. Particles of tissue were removed by sedimentation and filtration through a thick pad composed of glass-wool and cotton. To the dark brown liquid obtained was added a definite excess of HCl so that the mixture was quite acid. A creamy yellow precipitate was obtained which usually flocculated readily. Sedimentation, however, was assisted by the addition of alcohol. The purpose of the excess acid was to redissolve a portion of the protein, which is precipitated in slightly acid solution. After thorough washing with acid-alcohol, it was dried by repeated treatments with absolute alcohol and finally in a desiccator over fused $ZnCl_2$. The preparations obtained in this way were creamy to very light brown in colour, readily ground to a powder, but did not readily dissolve even in alkali. An intense dark blue colour was given with iodine. The ash content varied according to the completeness of the washing with acid-alcohol, but was usually quite low. The nitrogen content varied from 3 to 7 %.

(2) *Attempted purification of crude material.* Many methods of purification were tried and found to be unsuccessful. Precipitation with copper by the addition of Fehling's solution removed only a small portion of the admixed protein. Enzymic digestion both with pepsin and trypsin at the appropriate p_H lowered the nitrogen content somewhat; the best preparation, however, at the end of 10 days' treatment with pepsin still contained 1·9 % N. Fractional precipitation by cautious addition of acid and by the use of protein precipitants under slightly acid conditions proved no more successful owing to the very similar precipitation properties of the protein and carbohydrate.

More successful attempts were made at precipitation under alkaline conditions since the proteins are thereby kept in solution. To a filtered 2 % alkaline extract was added 1 volume of 90 % alcohol, whereupon a white flocculent precipitate appeared. This was filtered off, washed by sedimentation, first with alkaline alcohol to remove protein and then with acid alcohol to remove inorganic salts. It was finally dried with increasing concentrations of alcohol and over fused $ZnCl_2$ in a vacuum. The preparation contained ash, 0·57 %, nitrogen, 0·84 % and yielded sugar equivalent to 92·9 % anhydroglucose on hydrolysis with N H_2SO_4 for 16 hours. It was by far the purest preparation obtained up to this point. To the filtrate from it was added a further 2 volumes of alcohol and the white flocculent precipitate washed as before and dried. It was found to contain even less N, the ash being 1·36 % and N, 0·48 %. These two fractions, however, do not represent all the carbohydrate in the extract. This method of separation proved satisfactory in practice when amended by accomplishing the initial precipitation with at least 2 volumes of alcohol. In this way one fraction only is obtained, since hydrolytic studies had indicated the similarity in all but nitrogen content between the fractions precipitated by 1 and 3 volumes of alcohol respectively. This fraction will be referred to as Fraction B and probably corresponds to the callose of Mangin [1890].

(3) *Properties of Fraction B.* From fresh tissue preparations can be obtained which are colourless and dry readily to a fine powder. Once dried, they only redissolve with difficulty in alkali even on warming. This fraction was insoluble in water and acids and gave an intense blue coloration with iodine. After hydrolysis with acid, glucose was the only sugar detectable. The specific rotation could be determined in alkaline solution. (A solution of concentration 0·44 % in 3 % NaOH had a rotation $[\alpha]_D = +85{\cdot}1°$.) All attempts to remove the small amount of nitrogen still remaining have failed. That it is combined protein is possible, but not probable, in view of the variation in nitrogen content of preparations obtained by similar though not identical means. These have varied from 0·48 to 1·53 %. It was hoped that repeated precipitation from alkaline solution by 1 volume of alcohol might yield a product free from nitrogen, but this process does not seem to be effective and has a definite disadvantage in that the repeated re-solution in alkali, which is necessary, adversely affects the colour and texture of the precipitate. The best preparations are apparently those obtained by an initial precipitation with alcohol, washing by sedimentation first with alkaline alcohol of similar strength, and then with acid alcohol to remove inorganic salts. The possibility of the nitrogen being present not as protein, but as a hexosamine constituent of the carbohydrate, was considered. 5 g. of a preparation containing 1·53 % nitrogen were hydrolysed with concentrated HCl for 3 hours, diluted and the HCl distilled off under reduced pressure. After decolorising with norite the brownish solution was concentrated to a very small volume, cooled and allowed to stand. A small dark sediment appeared. This was filtered off, redissolved in a small quantity of water, and reprecipitated by the addition of dioxan. A very small amount of a white precipitate was obtained insufficient in amount for characterisation, but it was not hexosamine since it exhibited no reducing power in alkaline-copper solution. It would seem therefore that the nitrogen found in Fraction B is due not to hexosamine but solely to protein.

(4) *Hydrolysis of Fraction B.* (*a*) *By acids.* The hydrolysis of this polysaccharide fraction is readily accomplished by boiling with *N* H_2SO_4; less concentrated solutions did not give as complete hydrolysis even on boiling for prolonged periods. While the curves obtained for liberation of reducing sugar from various preparations are similar in general shape, curious differences were noted in the rate. The source and treatment of the preparation seemed inexplicably to affect the rapidity of hydrolysis. Some typical figures are given in Table III. The sample hydrolysing most rapidly and completely was No. 1, which had the lowest nitrogen content. This particular sample was physically in the best condition and on boiling with acid gave an opalescent and "starchy" solution. The other samples on boiling did not go uniformly into solution, even if previously ground and sieved so as to pass a 100-mesh sieve, but gave a more or less brownish suspension from which particles would separate out on standing. It seems reasonable to conclude that the presence of protein modifies the hydrolytic action for physical reasons. Some indication

of the correctness of this assumption was obtained by comparing the rate of hydrolysis of two samples of the same preparations, the one suspended in *N* acid and immediately boiled, while the other was previously boiled for 18 hours with water before bringing up to the same acid concentration. These two samples are respectively 3 i and 3 ii in Table III and it will be seen that the aqueous pretreatment by assisting penetration of the acid favourably influenced the rate of hydrolysis in the opening stages.

Table III. *Acid hydrolysis of alkali-soluble carbohydrate with* N H_2SO_4.

Preparation No.	Source and tissue	N content %	% reducing sugar calc. as glucose. Time of boiling in hours: 0·5	1	1·5	2	3	4	5	6	7	8	9	11	13	16	24
1	Fresh	0·84	61·0	79·5	83·0	85·3	—	—	96·3	102·0	102·5	—	—	—	—	103·3	—
2	Fresh	1·45	35·7	47·7	59·6	—	67·0	70·6	74·1	76·0	—	78·0	—	—	—	—	—
4	Fresh	1·6	34·7	48·5	—	57·2	58·3	—	72·8	—	—	73·9	—	—	79·1	—	—
3 i	Alcohol-extracted	1·53	24·2	39·4	47·6	—	52·0	58·6	—	—	68·7	—	—	73·2	—	—	—
3 ii	Alcohol-extracted	1·53	30·3	41·0	—	46·3	53·9	59·6	—	67·4	—	—	74·5	—	—	—	76·3
5	Fresh	0·95	42·2	56·0	—	71·7	74·6	—	80·8	—	80·8	—	—	—	—	—	81·1

The percentage recovery from these fractions on hydrolysis is far from satisfactory in most cases. This may be due to the presence of some undetected constituent unit, or possibly to incomplete hydrolysis owing to the presence of protein as suggested above. That the latter is the more probable explanation is indicated by the fact that the preparations with the lowest nitrogen contents, namely 1 and 5, gave the most complete recovery.

The only sugar to be identified in the hydrolysis liquid was glucose, characterised by means of its osazone. Tests for mannose, galactose, pentose, and uronic acid were negative. Confirmatory evidence for the presence of glucose was obtained by determining the rotation of the sugar obtained on hydrolysis as described earlier. A solution containing 0·0258 g. sugar per cc. calculated as glucose had a rotation of + 2·59° in a 2 dm. tube, $[\alpha]_D = + 50{\cdot}2°$, a close approximation to the theoretical for glucose.

Some information as to the type of linkage in the molecule was obtained by employing the method of interrupted hydrolysis developed by Norman [1929] in work on gums. The hydrolysis of all these preparations of Fraction B is most rapid in the first hour or so, after which the rate falls off sharply. Accordingly a sample of preparation 2 was taken and boiled for 1 hour with *N* H_2SO_4. Sugar equivalent to 42 % anhydroglucose was liberated. Three volumes of alcohol were added to the hydrolysis liquid at this point and the precipitate of partly hydrolysed material filtered off, washed with acid alcohol, and dried. This again was hydrolysed with *N* H_2SO_4. It was found that the initial rate of liberation of sugar was distinctly less than that of the original sample. Except for a slight lag due to the physical condition, the curve obtained coincided almost exactly with that of the original sample after 1 hour

of hydrolysis. These results are shown in Fig. 1. Curve 1 is the normal hydrolysis curve for the original sample, while curve 3 represents the figures obtained from the hydrolysed sample recalculated on the basis of the 58 % remaining at the end of the preliminary hydrolysis and plotted from the 1 hour point at which hydrolysis was recommenced. The close similarity between this curve and that of the original material is thereby apparent.

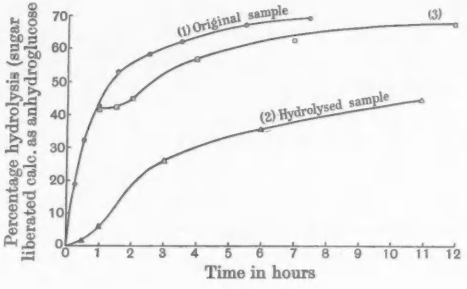


Fig. 1.

The inference therefore is that this polysaccharide contains two types of sugar linkage, one readily hydrolysed by *N* H_2SO_4, and the other more resistant.

(*b*) *By enzymes.* The hydrolysis of this polysaccharide by enzymes was also investigated. A preparation containing 1·45 % N was ground up to pass a 100-mesh sieve. Four samples of 0·5 g. each were taken and suspended in 50 cc. of water and boiled for a short time. After cooling, 10 cc. of a 2 % enzyme solution were added to each and the mixtures incubated in the presence of toluene. From time to time small aliquots were withdrawn, filtered and the reducing sugar present determined. The following enzyme

Table IV.

	Sugar liberated calc. as glucose (%)	
Enzyme	24 hours	96 hours
Malt diastase	16·9	17·3
Takadiastase	27·6	39·4
"Luizyme"	19·9	22·8

preparations were employed: (*a*) "pangestin," 37°, (*b*) malt diastase, 53°, (*c*) takadiastase, 53°, (*d*) "luizyme,"[1] 37°. The activity of the enzymes was verified under identical conditions on starch. The first named had practically no action on the suspended mould carbohydrate, but the remainder all caused the liberation of some reducing sugar, due correction being made for sugar from the enzyme preparation itself.

[1] A commercial product believed to be obtained from fungal material.

The actual nature of the sugar liberated has not as yet received attention. It is significant that takadiastase and "luizyme," both enzymes prepared from *Aspergillus oryzae*, are active in digesting this mould carbohydrate, but it was somewhat surprising to find that a malt diastase preparation should be active.

(5) *Preparation and properties of Fraction C.* If to the alkaline-alcoholic filtrate from the precipitation of Fraction B acid were slowly added, a cream-coloured flaky precipitate appeared at about p_H 6·2. Further acidification did not cause the separation of any more material, though the solution exhibited a very strong buffer effect at about p_H 4·7. The precipitate obtained at p_H 6·2 had a greyish-brown colour when dried and consisted largely of protein. The nitrogen content was between 10 and 11 %, indicating about 66 % protein. Glucose can be obtained on hydrolysis with *N* H_2SO_4 indicating the presence of a polysaccharide. Whether this is in combination with protein or not remains to be seen. The fact that the nitrogen content is rather consistent in different preparations might suggest that such is the case. This fraction has not been further investigated.

Summary.

1. An examination has been made of the carbohydrates of *Aspergillus fischeri* grown on a glucose-ammonium nitrate medium. But for a trace of pentose, the only sugar units detectable were glucose.

2. The chief water-soluble carbohydrate was precipitated by acid-alcohol. It gave no colour with iodine, was readily hydrolysed by boiling with 3 % H_2SO_4 and passed slowly through a cellophane membrane. It had a rotation of $[\alpha]_D^{22^\circ} = +86{\cdot}2^\circ$. Glucose only was obtained on hydrolysis.

3. Crude alkali-soluble preparations containing a high percentage of protein were obtained by extraction with alkali and precipitation with acid. Attempts at purification, by copper precipitation, enzymic digestion and fractional precipitation under acid conditions were unsuccessful.

4. By precipitation with alcohol from alkaline solution a carbohydrate of low nitrogen content was obtained. It gave an intense blue colour with iodine, and had a specific rotation of $+85{\cdot}1^\circ$. The residual nitrogen was present as protein, not as hexosamine. Takadiastase, malt diastase and "luizyme" all liberated reducing sugar.

5. An interrupted hydrolysis indicated the presence of two types of sugar linkage, one easily hydrolysed and the other more resistant. Glucose only was obtained on hydrolysis.

6. The filtrate from the preparation of this alkali-soluble fraction yielded on acidification precipitates containing about 66 % protein and the remainder carbohydrate. These have not been investigated.

REFERENCES.

Birkinshaw, Charles and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 355.
—— and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 331.
Boas (1917). *Biochem. Z.* **78**, 308; **81**, 80.
Cramer (1894). *Arch. Hyg.* **20**, 197.
Dox and Neidig (1914, 1). *J. Biol. Chem.* **18**, 167.
—— —— (1914, 2). *J. Biol. Chem.* **19**, 235.
Mangin (1890). *Compt. Rend. Acad. Sci.* **110**, 644.
Norman (1929). *Biochem. J.* **23**, 524.
Peterson, Pruess, Gorcica and Greene (1932). *Ind. Eng. Chem.* (In press.)
Raistrick *et al.* (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 136.
Schmidt (1925). *Biochem. Z.* **158**, 223.
Takata (1929). *J. Soc. Chem. Ind.* Japan, **32**, 245 B.
Thomas (1928). *Amer. J. Bot.* **15**, 537.
—— (1930). *Amer. J. Bot.* **17**, 779.
Winterstein (1894). *Z. physiol. Chem.* **19**, 521.

CCXXXI. THE CHEMISTRY OF MOULD TISSUE.
II. THE RESISTANT CELL-WALL MATERIAL[1].

By ARTHUR GEOFFREY NORMAN
and WILLIAM HAROLD PETERSON.

From the Departments of Agricultural Chemistry and Agricultural Bacteriology of the University of Wisconsin, Madison, U.S.A.

(*Received October 19th, 1932.*)

The cells walls of fungi lack the structure exhibited by those of higher plants, and at the same time are markedly inert towards the action of most reagents and stains. Many histological investigators, towards the close of the last century, occupied themselves with speculating on the nature of the cell-wall constituents. Tests for the usual structural constituents of plants were indefinite. Most of the workers assumed cellulose to be present, though so altered by combination or infiltration with other materials as to be difficult of characterisation. Several names were given to the cell-wall substance, the most general being "fungus cellulose." A chemical examination of this substance was made by Winterstein [1894; 1895] who showed it to be chitinous in nature. His investigations were mainly on material from higher fungi and his analysis showed about 3·6 to 3·9 % nitrogen in his preparations. From *Boletus edulis* Schöll [1908] obtained, by exhaustive extractions with hot 10 % KOH solution, a material which he described as pure chitin, yielding on hydrolysis 78 % of crystalline hexosamine hydrochloride. He concluded that the cell-walls of this organism are either composed of pure chitin or of chitin in very loose combination with a nitrogen-free carbohydrate. Later Sumi [1929] obtained chitin from the walls of spores of *Aspergillus oryzae*. He considered it to be present there in association with other substances. Proskuriakow [1926] has prepared from *Boletus*, *Agaricus* and *Polyporus*, by repeated extraction with 10 % NaOH followed by treatment with a solution of potassium permanganate, preparations of high nitrogen content (6·1–6·3 %). The actual amount of such material in the fungi is apparently small, since the yield from *Agaricus* was 5 % and from *Polyporus* 3–5 %. Takata [1929] by similar means obtained from *Aspergillus oryzae* 3·18 % of "chitin" containing 6·37 % N.

The purpose of these investigations was to examine the cell-wall material from *Aspergillus fischeri*, in comparison with that from higher fungi.

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

Experimental.

Preparation.

After prolonged alkaline extraction of the tissue, a cream-coloured residue remained. This after careful washing and dehydration with alcohol and ether dried to a colourless mass and was readily powdered. If dried without alcoholic dehydration, or if this treatment was not exhaustive, the residue on drying became grey or brownish in colour, hard and vitreous in character and almost impossible to grind to a powder. The nitrogen content of this material varied according to the violence of the alkaline treatment employed, but was in general rather under 3·5 %. Some figures obtained for nitrogen content and the treatment employed are given below. These are not all however from the same batch of mould mycelium.

	% nitrogen	% ash
1. Boiled with 60 % NaOH for 1 hour	2·69	0·26
2. Boiled with 25 % KOH for 2 hours	2·78	—
3. Boiled twice with 10 % NaOH for 48 hours each time ...	3·10	0·59
4. Boiled with 5 % NaOH for 3 hours after pre-treatment with more dilute alkali	2·41	1·22
5. Boiled with 4 % NaOH for 1 hour	2·35	—
6. Boiled with 2 % NaOH for 3 hours	2·53	0·37

Tissue, which had been previously treated with a fat solvent, saponified with alcoholic NaOH and then exhaustively extracted with hot NaOH solution yielded about 20 % of this resistant fraction. When such a preparation was boiled for 2 or 3 hours with concentrated HCl, diluted and concentrated under reduced pressure to remove HCl, decolorised with norite and again concentrated to a small volume, crystals separated out which were identified as hexosamine hydrochloride. (N found, 6·45 %; theoretical, 6·49 %.) With phenylhydrazine the crystals gave glucosazone, and they titrated nearly quantitatively as glucose with the Shaffer-Hartmann alkaline copper reagent. (1·005 mg. hexosamine hydrochloride titrated as 0·805 mg. glucose; theoretical, 0·835 mg. glucose.)

The presence of hexosamine in the resistant fraction of the cell-wall of fungal mycelium has long been recognised and was, of course, responsible for its description as "chitin." Its identity with chitin from animal sources, however, must not be assumed. The nitrogen content of all these preparations was about 3 %; even after the most violent alkaline treatment, namely by boiling for 4 days with 10 % NaOH, it was not increased above 3·1 %. Chitin from animal sources contains over 6 %. The fungal preparations of Proskuriakow [1926] similarly contained over 6 % N. Since anhydrohexosamine ($C_6H_9O_4.NH_2$) has a nitrogen content of 8·7 % it is evident that the resistant complex in the case of *Aspergillus fischeri* is only in part composed of hexosamine units. A nitrogen content of 3·0 % would indicate only 34·5 % anhydrohexosamine. The nature of the remainder of this complex has not been determined. Some workers have referred to the presence of cellulose in

the cell walls of fungi, and Thomas [1928] claims to have demonstrated its presence in *Fusarium*. His data, however, seem hardly sufficient, since materials other than cellulose are soluble in ammoniacal copper solution. The evidence to be presented below indicates that cellulose, as ordinarily found in plants, is not present. Combination with hexosamine units, of course, might possibly so alter its character and properties as to make it unrecognisable, but even this is unlikely in view of certain results obtained on hydrolysis with dilute acids.

Properties.

This resistant residue was wholly insoluble in Schweitzer's cuprammonium solution and in a saturated solution of zinc chloride in HCl, both solvents for cellulose. When ground to pass a 100-mesh sieve it was, however, readily and completely soluble in 72 % H_2SO_4 in the cold giving a slightly brown liquid. A xanthate was obtained by stirring in CS_2 after treatment with 17 % NaOH solution in the cold. The xanthate had a typical golden-brown colour and gave a smooth thick paste. On dilution and acidification the original material separated out. It had, however, no tensile strength either as a film or as a fibre. This residue was carefully washed with water and dried with repeated changes of alcohol. The nitrogen content was found to be unchanged by the process.

Acetylation.

In order to demonstrate the identity of celluose, should it be present in this fraction, several attempts were made at acetylation. 10 g. of tissue (N, 2·56 %) ground to pass a 100-mesh sieve were treated with 37·5 cc. of glacial acetic acid, 37·5 cc. of acetic anhydride and 1 cc. conc. H_2SO_4 as catalyst. After stirring for 2 minutes, the mass was allowed to stand for 30 minutes in a cold water-bath. The solution was then again stirred for 4·5 hours, diluted with 100 cc. glacial acetic acid and filtered. Very little appeared to have gone into solution. The clear filtrate was poured into 500 cc. distilled water and allowed to stand for several hours. A slightly milky emulsion was obtained on the addition of chloroform. The chloroform layer on separation was concentrated in the presence of a little water, upon which a granular white precipitate appeared in the water. This was centrifuged, washed several times in water and dried in a vacuum oven at 60°. The yield was very small, amounting to only 0·2 g. The acetyl content was determined by the method of Murray, Staud and Gray [1931] by solution in pyridine and treatment with NaOH at 53° for $\frac{1}{2}$ hour. (CH_3CO obtained 36·1 $\pm$ 1·5 %; theoretical for diacetylhexose anhydride, 34·9.) The residue was thoroughly washed and then suspended in water and extracted in a continuous extractor with *iso*propyl ether in order to remove all traces of free acetic acid. After drying with alcohol in a vacuum oven at 60°, the acetyl content was determined and found to be 5·8 %.

Acetylations were repeated under different conditions in an attempt to

obtain a product of higher acetyl content. By treatment of 10 g. of the resistant material with 0·5 g. $ZnCl_2$, 40 cc. acetic anhydride and 40 cc. glacial acetic acid at 50° for 24 hours with continuous stirring, an insoluble product containing 6·02 % acetyl was obtained, but no soluble product as in the first experiment.

Barnett's [1921] method as modified by Irvine and Hirst [1922] with another sample gave an insoluble product containing 10·7 % acetyl, but only a trace of soluble product. This method involves the use of $SOCl_2$ as a catalyst, the temperature being maintained at 65° for 18 hours, and is the most effective method for acetylation of cellulose, which, under these conditions, goes readily into solution and gives the triacetate. Since no soluble product was obtained, the conclusion seems justified that cellulose in its normal condition was absent. No attempt was made to determine the position of the acetyl groups in the acetylated material. Even on the hypothetical basis of the formation of a mono-acetyl anhydrohexose or hexosamine, calculation shows that there has only been the introduction of one acetyl group for every two hexose or substituted hexose units in the molecule. This resistant structural material is clearly not easily acetylated.

Attempts were made to isolate substituted fission products by acetolysis. 10 g. tissue were treated with 40 cc. acetic anhydride and 5 cc. conc. H_2SO_4 in the cold, and allowed to stand at room temperature for prolonged periods. It was not found possible to isolate any product. In the cold the material failed to go into solution, and if warmed, charring and decomposition occurred.

Hydrolysis.

As ordinarily obtained, the ash of this material was very low, while the nitrogen present indicated from 33–40 % anhydrohexosamine. Hydrolysis can be effected with concentrated H_2SO_4, but the recovery of reducing sugar expressed as glucose is far from complete. The hydrolyses were carried out as follows. About 0·5 g. of material passing a 100-mesh sieve was stirred with 10 cc. 80 % H_2SO_4 till completely dissolved, the beaker being kept cold during this process. After standing for varying periods in an ice-box, it was diluted to 200 cc. and the solution then autoclaved at 15 lb. for 1 hour. Reducing sugar was determined by the Shaffer-Hartmann method. Although the hydrolysis conditions were varied extensively, the highest yield of apparent sugar obtainable was 72 % calculated as anhydroglucose. The results obtained by this means were erratic, whereas good recovery can readily be obtained from cellulose. A small quantity of residue remained undissolved at the close of the hydrolysis. This was very difficult to determine accurately since washing was difficult and charring took place on drying. The amount varied from 5–10 %.

Acid hydrolysis under much milder conditions liberated appreciable quantities of reducing sugar. Table I indicates the percentage of apparent glucose from this resistant material on boiling with N and $N/2$ H_2SO_4.

Table I. *Hydrolysis of resistant cell-wall material.*

Time in hours	Percentage apparent glucose liberated		Time in hours	Percentage apparent glucose liberated	
	N H_2SO_4	$N/2$ H_2SO_4		N H_2SO_4	$N/2$ H_2SO_4
½	8·6	2·8	15	33·5	—
1	8·9	4·7	23	38·8	—
3	15·9	10·9	28	—	30·1
4	20·3	14·0	32	—	30·7
6	25·7	15·6	39	51·1	—
8	—	19·6	48	—	37·2

The nitrogen liberated during the course of this hydrolysis was estimated only at the end of 24 hours and 39 hours with N H_2SO_4.

Table II. *N liberated on hydrolysis with* N H_2SO_4.

Time in hours	Total N %	N liberated from 100 g. g.	Calculated as hexosamine
24	2·77	1·44	18·4
39	2·77	1·75	22·4

The important fact which emerges from these results is that both the nitrogenous (chitinous) part and the non-nitrogenous part of this complex are hydrolysed slowly by dilute acid treatment. In order to investigate more fully the relative rates of hydrolysis, a further series of experiments was carried out under pressure and with a number of acid concentrations. By this means the charring which took place on long boiling with acid was avoided, except in two or three cases when the autoclave treatment was prolonged. A weighed amount of material (0·5 g.) was placed in a tube with 30 cc. of acid of the required strength, autoclaved at 14 lbs., and then made up to 50 cc. After being allowed to settle, aliquot portions were taken for the determination of apparent glucose, and soluble nitrogen, subsequently calculated as hexosamine. The residue was then filtered through a Gooch crucible, thoroughly washed, dried and weighed. The original resistant material contained N 3·1 % and ash 0·59 %. The results are presented in Fig. 1.

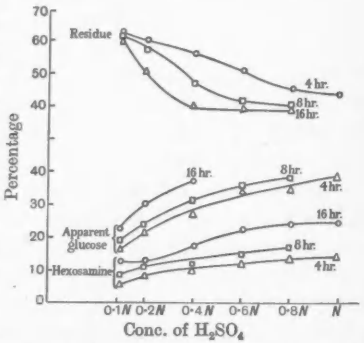


Fig. 1. Hydrolysis of resistant material with various strengths of H_2SO_4 at atmospheric pressure.

The evidence here presented indicates the very resistant nature of at least a portion of this residue; N H_2SO_4 at one atmosphere pressure removed 55 % in 4 hours and only an additional 8 % if the treatment was prolonged for a further 12 hours. Even then only 71·3 % of the nitrogen had been liberated. The original material on a basis of 3·1 % N contained 35·6 % anhydrohexosamine, while the residue at the end of 16 hours' treatment with N H_2SO_4

contained a very similar percentage, namely 34·7 % (1·12 g. N in 37·1 g. material). It should not, however, be deduced from this that the hydrolysis of the nitrogenous and non-nitrogenous units of this complex proceeded in parallel. This was definitely not the case for, in the early stages and under milder conditions, glucose or non-nitrogenous material was removed more rapidly than hexosamine. The approximate composition of the residue obtained throughout the 4-hour and 8-hour hydrolyses may similarly be calculated from the nitrogen not in solution. This is given in Table III. From this

Table III. *Approximate composition of hydrolysis residue.*

	4 hours				8 hours			
Concentration of acid	Residue %	Nitrogen in residue	Calculated as anhydrohexosamine	Anhydrohexosamine in residue %	Residue %	Nitrogen in residue	Calculated as anhydrohexosamine	Anhydrohexosamine in residue %
	45·1	1·98	22·7	50·3	—	—	—	—
0·8 *N*	46·2	2·0	23·0	49·9	41·5	1·72	19·8	47·7
0·6 *N*	52·0	2·13	24·5	47·2	43·0	1·86	21·4	49·8
0·4 *N*	57·1	2·30	26·4	46·2	47·8	2·13	24·5	51·3
0·2 *N*	60·7	2·44	27·1	44·7	58·5	2·20	25·3	43·3
0·1 *N*	63·5	2·67	30·7	48·4	62·3	2·44	28·0	45·0

it will be seen that practically one-half of the residue obtained in these two series was composed of anhydrohexosamine units. The remainder, however, cannot be considered as consisting solely of anhydroglucose units, though glucose was the only sugar detected as an osazone. The recovery throughout these hydrolysis experiments was far from complete and averaged about 80 %. By analogy with chitin from animal sources the presence of acidic groups was suspected, and this possibility was therefore examined. 1·362 g. of finely ground material were dissolved in 20 cc. 80 % H_2SO_4, allowed to stand for 3 hours in an ice-box and diluted to 400 cc. The solution was autoclaved for 1 hour and distilled in steam, 4 litres of distillate being collected. The distillate titrated equivalent to 29·4 cc. 0·103 *N* NaOH. The volatile acid was characterised as acetic by the Virtanen-Duclaux determination. The residue from the distillation of volatile acid was extracted in a continuous extractor with *iso*propyl ether for non-volatile acid. The extract when taken up with water contained only a trace of acid. It would seem, therefore, that acetic acid is liberated on hydrolysis in appreciable amount and must be considered as a constituent of the molecule. The sample of resistant material used in the above experiment contained 0·37 % ash and 3·55 % N; CH_3CO content, 9·55 %. The anhydrohexosamine content ($C_6H_9O_4.NH_2$) calculated from the nitrogen present was 40·8 %. Since, in the case of chitin from animal sources, the acetyl groups have been shown to be linked to the nitrogen of the glucosamine, it is not unlikely that a similar relationship may hold in this case. The acetyl content found corresponds very nearly to that required if there is one CH_3CO group for each hexosamine unit.

From this evidence obtained by hydrolysis it is not possible to make any very definite statement as to the nature of the resistant material. It is very improbable that a single entity is present. The changing ratio during hydrolysis between the hexosamine and the non-nitrogenous portion of the complex suggests the presence of two components hydrolysing at different rates. The fate of the acetyl groups in mild acid hydrolysis has not been determined, but the apparent production of a material containing about 50 % anhydrohexosamine with widely different concentrations of acid is significant. These hydrolytic studies provide further evidence for the absence of cellulose, which, since it would be unaffected by the low concentrations of acid employed, would cause the ratio of the nitrogenous to the non-nitrogenous part of the complex to fall rather than to rise, as was observed. Tentatively it is suggested that this resistant cell-wall complex has two components. The major one consists of one molecule each of hexosamine, glucose and acetic acid, the last probably being linked to the nitrogen as in animal chitin. The other consists only of glucose units, is more readily hydrolysed and is soluble to some extent in strong alkali. Their association may be compared to that between xylan and cellulose in many plant fibres.

Fermentation of the resistant residue.

Still further to investigate the possible presence of cellulose in this fraction, portions of it were fermented with cellulose-decomposing organisms of high activity. 200 g. of wet residue (26·6 g. dry matter) were suspended in 500 cc. of a mineral salt solution and 5 g. of $CaCO_3$ added. After prolonged sterilisation, a small quantity of sterile peptone was added (0·5 %) and the whole shaken and inoculated with a heavy inoculum of the desired organism. Two fermentations were carried out (A) with a "purified" culture of thermophilic cellulose-decomposing organisms—very active in cellulose fermentation but containing several forms, mainly short rods, (B) with a purer culture containing the cellulose organism isolated by Snieszko (unpublished work) together with its two concomitant contaminants, which together bring about a rapid and extensive decomposition of pure cellulose.

Both fermentation mixtures were incubated at 55° for a period of 3 weeks. The fermentation was obviously very slight. At the conclusion of this period the volume was adjusted and aliquots taken for determination of the unfermented residue. These were washed with dilute HCl to remove carbonate and then thoroughly with water. The fermentations were also examined microscopically to verify the cultures employed. The loss of organic matter was found to be slight; in A, 10·72 % and in B, 8·76 %. While this cannot be taken as evidence for the absence of cellulose, it at least indicates that it is present only to a small extent, if at all, for under similar conditions pure cellulose would have been almost completely decomposed by these cultures in a much shorter period of time.

SUMMARY.

1. After exhaustive alkaline treatment of the tissue of *Aspergillus fischeri*, about 20 % of a resistant cell-wall material remained.

2. The preparations yielded hexosamine hydrochloride on hydrolysis with HCl and had a nitrogen content of about 3 %. This indicated the presence of approximately 35 % anhydrohexosamine in the complex.

3. The material was insoluble in cellulose solvents, but gave a xanthate.

4. Only a trace of a soluble acetylated derivative was obtained; the insoluble product of acetylation contained 10·7 % acetyl. Acetolysis resulted in charring and decomposition.

5. Hydrolysis could be effected readily after solution in concentrated H_2SO_4, but the maximum recovery of apparent glucose was only 72 %. Dilute acid liberated both glucose and hexosamine slowly, and under pressure more rapidly. Glucose units were liberated more rapidly than hexosamine in the opening stages, and a residue containing about 50 % anhydrohexosamine was consistently obtained unless the treatment was violent.

6. Acetic acid was obtained on acid hydrolysis in amount approximately equivalent to one CH_3CO group for every hexosamine unit.

7. Fermentation with active cultures of thermophilic cellulose-decomposing organisms was slight, 10 % only being lost in 3 weeks.

8. The evidence suggests that cellulose, in the form usually met with in plants, is absent from this complex, which is probably a mixture of two components, one containing hexosamine, glucose and acetyl units, and the other, more readily hydrolysed, glucose units alone.

REFERENCES.

Barnett (1921). *J. Soc. Chem. Ind.* **40**, 8 T.
Irvine and Hirst (1922). *J. Chem. Soc.* **121**, 1585.
Murray, Staud and Gray (1931). *Ind. Eng. Chem.* (*Anal. Edit.*), **3**, 269.
Proskuriakow (1926). *Biochem. Z.* **167**, 68.
Schöll (1908). *Monatsh.* **29**, 1033.
Sumi (1929). *Biochem. Z.* **204**, 412.
Takata (1929). *J. Soc. Chem. Ind.* Japan, **32**, 245 B.
Thomas (1928). *Amer. J. Bot.* **15**, 537.
Winterstein (1894). *Z. physiol. Chem.* **19**, 521.
—— (1895). *Z. physiol. Chem.* **21**, 134.

CCXXXII. GLUCOSONE.

By KENDAL CARTWRIGHT DIXON and KENNETH HARRISON.

From the Biochemical Laboratory, Cambridge.

(*Received October 17th, 1932.*)

It has been suggested by Hynd [1927] that glucosone is an essential intermediary in carbohydrate metabolism and is partly responsible for the symptoms of insulin hypoglycaemia. The evidence on which these assumptions are based is not very satisfactory, and attempts have therefore been made to detect glucosone *in vivo* and *in vitro* under physiological conditions.

Experimental.

Preparation of glucosone. The original method of Fischer [1889] is lengthy, but a review of other methods by Hynd [1927] provides no good alternative. Evans, Nicoll, Strouse and Waring [1928] describe the production of glucosone by the oxidation of fructose with copper acetate, and a further method is described below.

Riley, Morley and Friend [1932] used selenium dioxide as an oxidising agent in the preparation of substituted glyoxals; they showed that fructose (but not glucose) reduced hot selenious acid, but they did not investigate the products formed.

A solution of 50 g. fructose and 15 g. selenious acid in 200 cc. water was heated for 3 hours under reflux on a boiling water-bath, and on cooling filtered from the reddish deposit of selenium. To the filtrate 70 g. lead acetate in 150 cc. water were added, and the solution after cooling in ice was filtered from the precipitated lead selenite. Ice-cold $Ba(OH)_2$ was now added until the solution was strongly alkaline, and the precipitated lead glucosone compound filtered off and washed thoroughly until free from fructose. From this stage the preparation now followed exactly that of Fischer [1889]. From the syrup so obtained (after purification by dissolution in alcohol and evaporation *in vacuo*) the osazone of glucose was prepared by warming to 37° with phenylhydrazine acetate for 30 minutes, filtering off and recrystallising from alcohol (see Table I).

Table I. *Melting-points.*

(*a*) Glucose phenylosazone from glucose	192–3° uncorr.
(*b*) Glucose phenylosazone from glucosone made by this method	193–4° „
(*c*) Mixture of (*a*) and (*b*)	192–3° „

Reactions of glucosone. Glucosone gives Schiff's reaction immediately in the cold, and is also bound by sodium sulphite; it therefore probably contains a free aldehyde group. A strong solution gives an immediate precipitate in the cold with 2:4-dinitrophenylhydrazine; the osazone (of glucose) formed crystallises in needles M.P. 252–3° (corr.); Glaser and Zuckermann [1927] give 256–7° "bei raschem Erhitzen." For the detection and estimation of glucosone in dilute solution, an equal volume of the reagent (0·5 g. 2:4-dinitrophenylhydrazine in 30 cc. 2*N* HCl [Case and Cook, 1931]) is added, and the solution allowed to stand at 37° for an hour; further standing is liable to bring down glucose and fructose if these are present. This reaction is delicate, a precipitate being given under these conditions with 0·03 % glucosone solution. Trichloroacetic acid does not interfere.

Glucosone is not appreciably destroyed by evaporation *in vacuo* provided the solution is acid. A small quantity of glucosone was dissolved in 100 cc. blood-serum, and the proteins precipitated with 5 g. trichloroacetic acid. The filtrate was made up to 100 cc., and 20 cc. were withdrawn (solution A). The remainder was evaporated *in vacuo* at 45° to a syrup, and again evaporated after the addition of 50 cc. water; the residue was diluted to 80 cc. and 20 cc. withdrawn (solution B). To solutions A and B 10 cc. of the 2:4-dinitrophenylhydrazine reagent were added, and after standing at 37° for an hour the precipitates were weighed:

Weight of osazone from A	...	...	31 mg.
Weight of osazone from B	...	...	29 mg.

The loss is not greater than the experimental error involved.

Attempted detection of glucosone. In the first series of experiments attempts were made to detect glucosone in the blood of rabbits in hypoglycaemic convulsions following a large overdose of insulin. Details are given in Table II. The insulin was injected intravenously unless otherwise stated.

Table II.

Rabbit No.	Weight kg.		Insulin units	Time of onset of convulsions hours*	Blood obtained cc.
1	2·1		26		
		1 hour later	26	2	20
2	2·8		20†		
		1 hour later	20	4	25
3	2·7		6		
		1 hour later	6	3	15
4	2·2		6		
		1½ hours later	6	1½	10
5	3·3		Control		30

* As from the second injection. † Subcutaneous.

When convulsions occurred the animals were killed by a blow on the head, and rapidly bled from the neck into 10 cc. 100 % (w/v) trichloroacetic acid. The filtrate and washings from the precipitated proteins were evaporated

in vacuo at 45° to small bulk, and an equal volume of 2:4-dinitrophenylhydrazine reagent was added. In no case was any precipitate formed after an hour at 37°. In all cases a deep red coloration, more marked in the insulinised animals than in the control, was observed on adding the reagent; this may be due to the presence of glyoxals other than glucosone. Rabbits 1 and 2 were fed on carrots for 24 hours previous to injection; 3 and 4 were starved for 48 hours previously, while the control was fed normally.

Since these experiments gave a negative result, a further attempt was made to detect the formation of glucosone from insulin and fructose in the presence of liver tissue. The technique employed was essentially that of Neuberg and Gottschalk [1924] and Gottschalk [1925] in a similar investigation. The following flasks were set up:

	A	B	C	D
Liver, g.	100	100	100	100
Calcium sulphite, g.	4	4	4	4
Fructose, g.	2	2	2	2
Insulin, units	40	—	40	—
Antiglyoxalase, mg. (Parke, Davis pancreatin)	—	—	20	20

To each flask were added 100 cc. phosphate buffer at p_H 7·0 and 45 mg. 8-hydroxyquinoline sulphate (neutralised with NaOH) as antiseptic. The flasks were incubated at 37° for 24 hours, a slow stream of air being passed through to stir the contents and render the system aerobic. After deproteinising with trichloroacetic acid, the filtrate and washings were evaporated to small bulk *in vacuo*, 50 cc. water added and again evaporated; any methylglyoxal or acetaldehyde present was thus removed. The solutions were filtered, diluted to 20 cc. and after adding 10 cc. of the reagent were allowed to stand at 37° for an hour. No precipitation occurred.

Discussion.

From the evidence available it does not seem that glucosone plays any important part in intermediary metabolism. The evidence of Hynd [1927] rests on a superficial similarity between the convulsions of insulin hypoglycaemia and those produced by glucosone: it was also found that adrenaline and pituitrin exerted an antagonising influence in both cases. The injection of other glyoxals, however, also causes convulsions: thus Kermack, Lambie and Slater [1927] found that methylglyoxal, and again [1929] that hydroxypyruvic aldehyde were toxic, though the last observation has been disputed by Hynd [1931]. Moreover, a very large dose of glucosone is required to produce the effect; Herring and Hynd [1928] found that 1·7 mg./g. body weight was the convulsant dose for rabbits: calculation shows that the glucosone must be present in the blood (assuming that it is not immediately removed) to the extent of more than 1 %. Unless, therefore, glucosone disappears from the blood very rapidly, the failure to detect it in appreciable quantities (more than 0·01 %) renders it unlikely that the convulsions of insulin hypoglycaemia

are even partly due to its presence. Evidence that glucosone is not rapidly removed from the blood was found by Hynd [1927]; 15 minutes after the injection of a small quantity of glucosone into a mouse, the apparent blood-sugar (determined by MacLean's method, which is affected by glucosone) had risen from 0·161 % to 0·238 %. Kermack, Lambie and Slater [1929] have pointed out that the absence of convulsions when glucose is injected along with insulin renders it improbable that glucosone causes these convulsions. Further, it is curious that the injection of glucose should alleviate the convulsive condition, since in a system glucose $\xrightarrow{\text{insulin}}$ glucosone the increase in the concentration of glucose should increase the production of glucosone correspondingly.

The attempted isolation of glucosone from incubated liver tissue also failed. Antiglyoxalase was added to flasks C and D to prevent the oxidation of glucosone, if it were formed, to gluconic acid, since experiments indicate that glyoxalase promotes this change. (Further work is in progress on this question, since it is contrary to the finding of Levene and Meyer [1915].) There is thus no evidence that glucosone is formed by the liver, even with added insulin; Clift and Cook [1932, private communication] have shown that glucosone does not occur in fresh liver, and we have confirmed this result. It is useless to attempt to account for the discrepancy which has been pointed out [Holden, 1926 and others] between the reducing power and rotation of blood filtrates by the presence of glucosone, since Groen and Meyer [1932] have shown that reduced glutathione is responsible, even in the case of diabetic patients under insulin treatment. The possibility of glucosone acting as an intermediary cannot be excluded entirely, since Walker [1932] has observed that it is formed from glucose by a mould of the *flavusoryzae* group of *Aspergilli*; it has not been found possible, however, to detect it in the animal body.

Summary.

1. A method is outlined for the preparation of glucosone from fructose by oxidation with selenious acid; a method for its detection is also described.

2. Glucosone does not occur to the extent of more than 0·01 % in the blood of rabbits suffering from convulsions caused by insulin, and it seems that this condition is therefore due to the lowered blood-sugar alone, and not to the accumulation of glucosone.

3. No evidence was obtained that glucosone is formed by the liver, even in the presence of insulin, nor does it occur in fresh liver.

4. There is thus no evidence that glucosone is formed in the intermediary metabolism of the animal body.

We wish to express our thanks to Sir F. G. Hopkins, to Dr Hamilton McCombie and to Dr T. S. Hele for their advice and criticism; and to Mr J. O. Giršavičius and Mr F. P. Clift for a number of suggestions.

REFERENCES.

Case and Cook (1931). *Biochem. J.* **25**, 1319.
Evans, Nicoll, Strouse and Waring (1928). *J. Amer. Chem. Soc.* **50**, 2667.
Fischer (1889). *Ber. deutsch. chem. Ges.* **22**, 87.
Glaser and Zuckermann (1927). *Z. physiol. Chem.* **167**, 37.
Gottschalk (1925). *Biochem. Z.* **155**, 348.
Groen and Meyer (1932). *Biochem. J.* **26**, 624.
Herring and Hynd (1928). *J. Physiol.* **66**, 267.
Holden (1926). *Biochem. J.* **20**, 263.
Hynd (1927). *Proc. Roy. Soc. Lond.* B **101**, 244.
—— (1931). *Biochem. J.* **25**, 11.
Kermack, Lambie and Slater (1927). *Biochem. J.* **21**, 40.
—— —— —— (1929). *Biochem. J.* **23**, 410.
Levene and Meyer (1915). *J. Biol. Chem.* **22**, 337.
Neuberg and Gottschalk (1924). *Biochem. Z.* **146**, 164.
Riley, Morley and Friend (1932). *J. Chem. Soc.* 1875.
Walker (1932). *Nature*, **130**, 582.

CCXXXIII. THE AUTOXIDATION OF DIALURIC ACID.

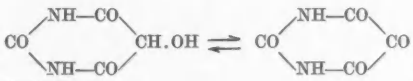
By GEORGE MAXWELL RICHARDSON.

From the Department of Physiology and Biochemistry, University College, and the Biochemical Department, Imperial College of Science, London.

(*Received October 20th, 1932.*)

Of recent years, biological studies of oxidation-reduction have been chiefly concerned with the rate and specificity of the oxidative reactions between metabolites. At the same time, many oxidation-reduction systems have been precisely defined electrometrically by measurements at an inert electrode. It will therefore be interesting to co-ordinate, where possible, the kinetics of oxidation with the oxidation-reduction potentials of the reacting systems.

With this end in view, a previous description of the electrode potentials of the dialuric acid-alloxan system:



included some preliminary data describing the atmospheric oxidation of dialuric acid [Richardson and Cannan, 1929]. The extent to which the reactants would associate to the dimeride alloxantin and interfere in the system was also formulated, but was found to be scarcely significant in dilute solution. The rapid, roughly linear velocity of oxidation obtained was therefore not an unlikely one. At the same time the manifestation in the data of some correlation in acid solution between electrode potential and rate of oxidation made an extended investigation using improved technique highly desirable.

While such experiments were being initiated with a Warburg gas absorption apparatus, the first of a series of three papers dealing with this autoxidation [Hill, 1930; 1931; 1932] appeared. His observations, however, would not submit to an orderly interpretation, and agreed neither with our published findings nor with such gasometric data as were then available. His calculations regarded alloxantin as the end-product of the oxidation, an assumption irreconcilable with the actual direct observations of Thunberg [1915]. Consequently, a much more thorough investigation of this autoxidation than was at first anticipated is now to be reported.

Experimental.

The rate of diffusion of oxygen into a reaction mixture effectively limits the technique available for measuring atmospheric oxidation velocities. In previous experiments we added solutions already saturated with oxygen to dialuric acid at high dilution to overcome this limitation, but the increased liability to error at such dilutions made a different technique desirable. It was also clear that oxidation in other than acid solution was too rapid to allow the accurate withdrawal of samples for titrimetric estimation of velocity. Yet current indicator methods were felt to be poorly suited to defining a continuous series of points during the oxidation. Consequently the gasometric measurement of oxidation in a set of six Warburg absorption manometers was adopted, small quantities of less dilute solutions being exposed to as large a surface of air as possible. Although the apparatus is not well suited to following oxidations with the rapidity that was attempted, the application of extensive controls should have minimised fortuitous errors. An abundance of fairly reproducible observations has been accepted as the practical criterion of reliability.

The preparations of dialuric acid, H_2O; alloxan, H_2O; and alloxantin, $2H_2O$ used were identical with those employed for the previous investigation. Stock solutions of dialuric acid and alloxantin in 0·05 *N* HCl were made up in sufficient quantity to last 2 or 3 days and were stored under hydrogen for immediate delivery from a burette into the absorption flasks. Solutions were approximately saturated at room temperature (0·025 *M* dialuric acid, and 0·0083 *M* alloxantin), and 2 or 3 times daily were titrated directly against 0·01 *M* iodine solution with starch indicator [*cf.* Biilmann and Bentzon, 1918]. Titratable reducing power never changed by more than 3·5 % on storing, and was seldom smaller than 95 % of the value calculated by weight. In the calculation of results, the iodine titre is accepted as the measure of dialuric acid concentration.

Stock buffer solutions adopted were 0·1 *M* and 0·05 *M* HCl, 0·1 *M* citrate, and Prideaux and Ward's universal buffer mixture 0·1 *N* with respect to each constituent group (this is of greater strength than is customary, care being necessary in cold weather lest the phenylacetic acid should become supersaturated in acid stocks). Buffer p_H values were measured with a Hildebrand hydrogen electrode, a saturated KCl calomel half-cell and a Cambridge portable potentiometer.

Experiments were conducted in the Warburg apparatus under the following conditions.

Temperature of thermostat, 18°; manometer liquid, Brodie's fluid (density $1{\cdot}032_2$); average capillary diameter 0·35 mm.; amplitude of swing, about 6 cm.; number of oscillations per min., about 120; capacity of flasks to manometer level, (1) 20·1 cc., (2) 19·5 cc., (5) 23·2 cc., (6) 20·3 cc. (by weight of mercury); volumes of experimental solutions, 2·4 and 1·2 cc.; average exposed

area of base of flasks 8·7 cm.^{2}; average depth of liquid, 2·8 and 1·4 mm. All flasks in use were of the same general dimensions, and were constructed in the usual conical shape with side-arm and NaOH tube.

For each experiment, the stock solutions were delivered directly into the flasks from micro-glassware. The proportions were decided so that the reacting mixtures should contain: dialuric acid of the required concentration, the exact equivalent of 0·1 *M* NaOH to bring the dialuric acid solution to the desired p_H, as large an excess of buffer solution as possible, comparable concentrations of total salts, added catalytic solutions where desired, and, finally, distilled water to the required volume. Table I describes the solutions used between p_H 4 and 11. At more acid p_H values, the proportions were suitably modified

Table I. *Constitution of reaction mixtures.*

Stock reductant: 0·025 *M* dialuric acid (or 0·0083 *M* alloxantin) in 0·05 *M* HCl.

	Conc. of dialuric acid					Conc. of alloxantin	
	0·0166 *M*	0·0125 *M*	0·0066 *M*	0·0063 *M*	0·0021 *M*	0·0055 *M*	0·0021 *M*
Reductant (cc.)	1·6	1·2	0·4*	0·6	0·2	1·6	0·6
0·05 *M* HCl (cc.)	—	—	—	0·6	1·0	—	0·6
0·1 *N* buffer (cc.)	0·6	0·75	0·5	0·75	0·75	0·6	0·75
0·2 *M* NaOH (cc.)	—	0·45	0·14	0·375	0·325	—	0·35
0·8 *M* NaOH (cc.)	0·15	—	—	—	—	0·133	—
Water + catalyst (cc.)	0·05	0	0·16	0·075	0·125	0·067	0·1
Total volume (cc.)	2·4	2·4	1·2	2·4	2·4	2·4	2·4
Buffer strength	0·025 *N*	0·031 *N*	0·042 *N*	0·031 *N*	0·031 *N*	0·025 *N*	0·031 *N*
Ionic strength (p_H 8)	0·15	0·16	0·19	0·16	0·15	0·14	0·15

* 0·02 *M* dialuric acid stock.

for concentration of HCl un-neutralised and of dialuric acid un-ionised at the desired p_H (dialuric acid $p_K = 2·8$). Where catalyses were attempted, the quoted catalytic strengths were obtained by dilution from stock solutions of 0·02 *M* ferrous sulphate in 0·005 *M* HCl, 0·1 *M* KCN (from 95 % KCN solid), and 0·01 *M* barbituric acid (all by weight). The acidity or alkalinity of the added solutions was included in estimating the NaOH additions required above.

In initiating an experiment the main body of the flask received the dialuric acid, HCl and distilled water, the other constituents being added to the side compartment. Thus dialuric acid solutions were stored at optimum stability awaiting immediate admixture after temperature equilibration. Where it was necessary, the NaOH tube received 0·05 cc. 40 % NaOH on filter-paper (of vol. displacement 0·025 cc.) as CO_2 absorbent. After temperature equilibration, the manometers were reset to the standard levels, the gas system closed, and the solutions mixed by tilting at zero time. The method, allowing the rapid admixture of fully aerated solutions not confined in narrow tubes, permits estimation of zero time to within a few seconds. It also obviates the presence of metal parts of doubtful catalytic inertness. At the same time the degree of vagueness as to final p_H which it certainly permits is overcome by the large number of experiments conducted.

The course of the reaction was followed by manometer readings at intervals appropriate to the rapidity of absorption, barometric fluctuations being ascertained from a control manometer. As the half-life period of dialuric acid varied from more than 75 min. down to 1 min., the faster reactions were necessarily followed only singly or in pairs. Nevertheless, certain observations obviously refer to rates outside the limits of effective measurement of the apparatus and can be traced as such in the data.

The maximum oxygen requirement for the formation of alloxan and H_2O_2 from the solutions is theoretically 0·9 cc. This is 24 % of the total oxygen of the flasks. Actually, however, unregulated initial oxidation will have somewhat diminished this requirement. Nevertheless, since the acid solutions were exposed to air before buffer admixture only for some 7 to 10 mins., during which time oxidation with even vigorous agitation will be found not to exceed 20 to 30 %, preliminary oxidation with the apparatus stationary cannot have been large.

Calculation of results.

Notation.

T = absolute temperature in ° C.

α_{18} = solubility of the reactive gas in cc. per cc. solution.

V_s, V_a, V_g = volumes in cc. of the solution, the CO_2 absorbent, and the gas space (empty), respectively.

C = a constant for each flask and each value of $(V_s + V_a)$.

h = alteration of pressure in mm. Brodie's fluid.

$X = hC$ = oxygen uptake in cc. (at 0° and 760 mm.).

n = g.-mol. oxygen absorbed per g.-mol. dialuric acid.

m = intended molarity of dialuric acid.

y = correction factor determined by iodine titration.

x = degree of oxidation $= \dfrac{X}{\text{theoretical } O_2 \text{ uptake}} = \dfrac{X}{22{\cdot}4 V_s nmy}$.

t = time in minutes.

$k = \dfrac{x}{t} = \dfrac{X}{22{\cdot}4 V_s nmyt}$ = linear velocity constant of oxidation.

Procedure.

In the Warburg (constant volume) method using Brodie's fluid in the manometer, the gas absorption is related to the pressure change by the following formula:

$$X = \frac{h}{10{,}000}\left[(V_g - V_s - V_a)\frac{273}{T} + V_s\alpha_{18}\right] = hC.$$

For flask No. 1, using 2·4 cc. solution and the adopted CO_2 absorbent, this reduces to:

$$X = \frac{h}{10{,}000}\left[(20{\cdot}1 - 2{\cdot}4 - 0{\cdot}075)\frac{273}{291} + 2{\cdot}4\,(0{\cdot}031^*)\right] = 1{\cdot}660h \times 10^{-3}.$$

* Since the partial pressures of nitrogen and water vapour are constant during the reaction, this term should comprise just the solubility of oxygen in the aqueous buffer. It is a small magnitude (0·4 %) in the whole expression. Since Randall and Failey's solubility correction [1927] for the variable ionic strength of the experimental solutions ($\mu = 0{\cdot}06$ to 0·23 according to p_H) is not greater than ± 3 % of α_{18}, it is not significant in these experiments.

When, during an experiment, a control manometer and flask containing 2·4 cc. water has been used to compensate readings of h for barometric fluctuations, the constant C becomes entirely independent of atmospheric pressure.

Observed pressure changes may therefore be converted directly into cc. of oxygen absorbed. In those cases where the manometers had been rapidly reset to cover pressure changes in excess of 250 mm., conterminous readings are simply totalled together, the point of resetting being appropriately indicated in the Figures and Tables. The oxygen uptake is then, after adjustment for y, plotted against time. Since the curve is approximately linear, the reaction may be defined directly by reading the appropriate absorption constant X/yt from the graph. Table II and Fig. 1 illustrate the procedure for a typical set of straightforward oxidations.

Table II. *Calculation of data. Variation of* k *with dialuric acid concentration.*

Citrate buffer; p_H 2·5.

m	0·0166 M		0·0125 M		0·0066 M		0·0063 M		0·0021 M	
V_s	2·4 cc.		2·4 cc.		1·2 cc.		2·4 cc.		2·4 cc.	
y	0·946		0·986		0·946		0·946		0·983	
Flask No.	(6)		(3)		(1)		(2)		(6)	
t (mins.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)	h (mm.)	X/y (cc.)
2	−22	0·039	−18	0·027	−6	0·011	−12	0·021	−3	0·005
4	51	0·091	44	0·066	17	0·032	29	0·049	9	0·016
6	86	0·153	78	0·117	29	0·054	52	0·088	15	0·026
8	120	0·214	111	0·167	43	0·081	76	0·129	21	0·036
10	151	0·269	148	0·223	55	0·103	99	0·168	27	0·046
13	**200**	**0·356**	**200**	**0·302**	67	0·126	132	0·224	36	0·062
16	240	0·427	242	0·364	70	0·131	151	0·256	42	0·072
20	298	0·530	302	0·455	,,	,,	159	0·270	41	0·071
25	346	0·616	333	0·501	,,	,,	158	0·268	,,	,,
30	357	0·635	332	0·500	,,	,,	,,	,,	,,	,,
35	359	0·639	,,	,,	,,	,,	,,	,,	,,	,,
40	360	0·641	,,	,,	,,	,,	,,	,,	,,	,,
X/yt (mean)	0·029		0·026		0·012		0·019		0·005	
k	0·032		0·039		0·067		0·056		0·044	
log k	−1·50		−1·41		−1·17		−1·25		−1·36	

The constant X/yt, however, describes only a volume change, and will not reflect the molecular extent of the oxidation until it is converted into k, the degree of oxidation per minute $\left(\frac{X}{yt} \times \frac{1}{22{\cdot}4V_snm}\right)$. Since $22{\cdot}4V_snm$ is a constant in any one reaction, the calculation of k follows directly. k is utilised in its logarithmic form for the ultimate tabulation against temperature, concentration, p_H, *etc.*

It might be well to indicate that comparative tabulation of log k is quite valid whatever value is postulated for n. The expression $\frac{1000X}{V_smyt}$ (cc. oxygen per g.-mol. dialuric acid per min.) involves no assumption about the reaction,

but differs from k only in the constant, 22,400n, which is characteristic of the reaction in general. In the comparative, as distinct from the absolute, evaluation of constants, no presumption is implicit as to the molecular proportions of the reaction.

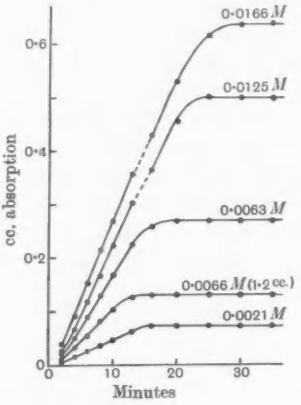


Fig. 1.

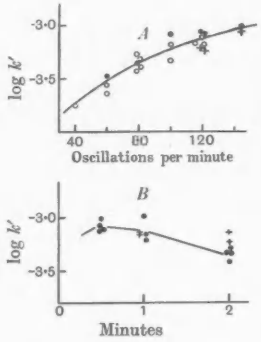


Fig. 3.

Fig. 1. Rate of absorption of oxygen. 2·4½cc. p_H 2·5. Citrate buffer. 18°.

Fig. 3. A. Effect of rate of oscillation. $k' = k\sqrt{m}\sqrt[n]{H^+}$. 2 min. readings. • p_H 7·9, + p_H 3·9 (1·2 cc., 0·0066 *M*. CO_2-free). o 0·005 and 0·01 *M* KCN (assorted). B. Effect of discontinuous oscillation. k' as above. 2·4cc. 0·0166 and 0·0125 *M*. CO_2-free. 120 oscillations per min. • p_H 7·9 and 9·0, + p_H 6·0.

Results.

Stoichiometric proportions.

If the autoxidation represents solely the production of alloxan, it should require two equivalents of oxidising agent, or 11·2 cc. oxygen per millimol. dialuric acid. In Fig. 2 the total oxygen absorbed experimentally is depicted, the included data being a selection, calculated as cc. oxygen per millimol. dialuric acid, from measurements which utilised not less than 0·03 millimol. dialuric acid in each experiment. The data differ in no significant fashion from those for the smaller amounts of dialuric acid. In those cases where the oxidation initiates CO_2 production, the CO_2 has been suitably removed. In Fig. 2 the maximum loss of reductant during temperature equilibration has also been inserted, after calculation from the rate of oxidation of dialuric acid at p_H 1·4 ($\log k = -1·6$, see later). It is clear that, with the possible exception of the region p_H 3 to 4, the molecular proportion oxygen/dialuric acid is undoubtedly double the expected ratio. In other words, the reactants are combining in equimolecular proportions, so that $n = 1$. This value is embodied in subsequent calculation of k.

Since it was probable that the technique adopted by Hill [1930; 1931] had measured only the later stages of the reaction, Fig. 2 gives a comparison with a recalculation of Hill's data upon the same basis. The probability is confirmed.

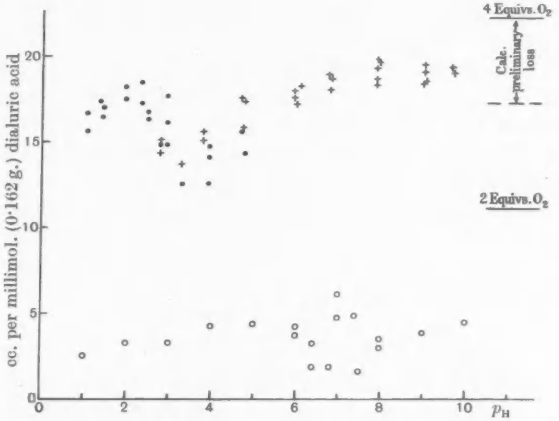


Fig. 2. Variation of total absorption with p_H. 2·4 cc. 0·0166 and 0·0125 *M*. • not CO_2-free, + CO_2-free, o data of Hill (10 cc., 0·018 and 0·0062 *M*).

Control of technique.

The rapidity of the reaction made it essential to determine the effect of varying technique upon the velocity measurements. In particular, such agitation of the reacting solutions was required that oxygen concentration would not be limited by its rate of diffusion. The rapidity of oscillation, therefore, was increased to the maximum speed of the apparatus by reducing the amplitude of swing to its limit (6 cm.). Unfortunately, this amplitude was still too great to permit the continuous reading of the manometers, and cessation of movement for a few seconds was ultimately necessary to obtain final readings after each approximate setting. Fig. 3 represents the definition of optimum conditions.

The data primarily represent alkaline oxidations, where it was expected that the speed of reaction would accentuate the possible inherent defects of technique. They represent a variety of values of p_H and m, and are therefore corrected to a common ordinate for convenient presentation by applying the conclusions of later sections. The separate portions of Fig. 3 represent (A) the conditioning of oxidation velocity by frequency of oscillation, and (B) the influence of discontinuous oscillation, interrupted at intervals of 2, 1 and 0·5 min., upon the observations. Since experiments, excepting those with added KCN, were conducted in the presence of a CO_2 absorbent, the efficiency of CO_2 absorption by 40 % NaOH on a cylinder of filter-paper must be known as a control. This has, however, already been examined by Dixon and Elliott [1930].

It is clear in section A of Fig. 3 that the rapid reaction is very considerably conditioned by the frequency of oscillation. It is probable that k' varies as some power of the frequency, and that, although an approximate maximum was achieved at the adopted frequency of 120 complete oscillations per minute (limited by the inertia of the apparatus) faster absorptions would obtain at greatly accelerated frequencies. The increase within realisable frequencies, however, would not greatly exceed the somewhat generous experimental error. The inserted curve expresses the arbitrary relation $k' = C'$ (frequency)$^{1\cdot33}$. It may be noted that the entirely distinctive reaction which occurs in the presence of 0·01 *M* and 0·005 *M* KCN, yet shows an effect of the degree of agitation which does not differ from the effect in the absence of KCN. In section B, it is apparent that interruption of oscillation becomes significant only for intervals of shorter duration than 0·5 min., and that 1 min. readings record the rapid reactions more closely than 2 min. readings. The variability is not appreciably greater than experimental error.

The velocity of autoxidation.

Fig. 4 depicts the gas volume change in a set of direct observations on dialuric acid solutions of varying p_H. The curves are clearly the resultant of two separable effects, namely, the effect of p_H on the rapidity of oxygen absorption (apparent more clearly from p_H 1·1 to 2·0), and the effect evident between p_H 4·5 and 11 of some opposing volume change which culminates

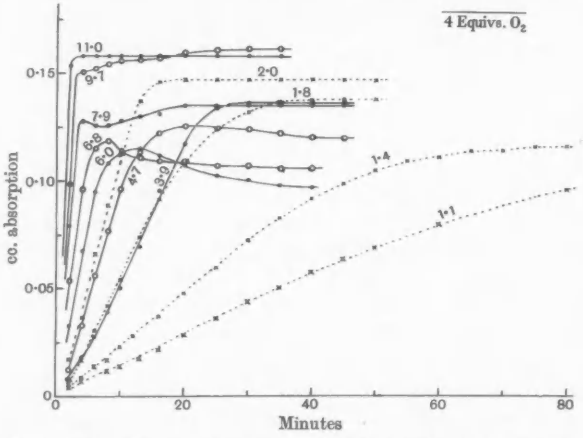


Fig. 4. Variation of absorption rate with p_H. 1·2 cc. 0·0066 *M*. Not CO_2-free. Universal buffer.

about p_H 6. The opposing reaction is slower in developing than is the oxidation, and is only able to reverse the volume change when oxidation is far advanced and somewhat slow. The effect is not large in extent, and would appear to have no appreciable influence on oxidation velocity during the

initial stage of the reaction. It is maximum between p_H 6 and 7. Fortunately it is completely eliminated by an effective CO_2 (or other volatile acid) absorbent, and under such conditions the measured volume change is, as has been noted in Fig. 2, closely similar to that of acid reactions and is in simple stoichiometric ratio with the dialuric acid present. All data now to be quoted, unless otherwise indicated, have been obtained in the presence of a CO_2 absorbent if alkaline to p_H 4·7. The further description of the opposing reaction is reserved for a subsequent paper.

Fig. 5 is an assembly of data obtained at varying p_H values and concentrations of dialuric acid, where each point represents a separate experimental

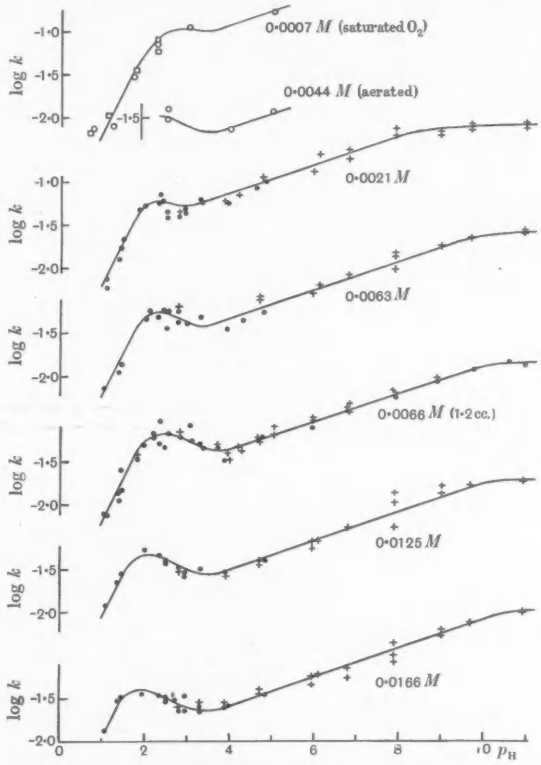


Fig. 5. log k:p_H curves. 2·4 cc. 18°. (Alkaline data by readings at 0·5 or 1 min. intervals.) • not CO_2-free, + CO_2-free, o iodimetric data, □ $TiCl_3$ data.

curve of the type depicted in Figs. 1 and 4. The agreement is not as poor as might have been expected from the limited adaptability of the apparatus for rapid oxidations, and is quite sufficient to describe the situation with little uncertainty. It is seen that the effect of p_H is separable into three regions,

first, p_H 1 to 2, a region of rapidly increasing reaction velocity, next, p_H 2 to 4, one of uncertain interpretation which, significantly, corresponds with the incomplete oxidations of Fig. 2, and then finally, on the alkaline side of p_H 4, a region of steady increase of velocity as the 5th or 6th root of $[H^+]$, terminated by velocities too rapid for measurement. Log k in the inserted curves varies inversely as $[H^+]$ from p_H 1 to 2, and above p_H 4 inversely as $\sqrt[6]{[H^+]}$.

The fragmentary data included for 0·0007 *M* and 0·0044 *M* dialuric acid solutions are those already referred to [Richardson and Cannan, 1929] and are now included in full for reference. The 0·0007 *M* solutions were oxidised in a closed chamber by saturated solutions of oxygen (final conc. O_2, *ca.* 0·0011 *M*) and the reaction followed titrimetrically in two ways: firstly, by depressing the autoxidation of appropriate 25 cc. samples with excess HCl and immediate titration under nitrogen with 0·005 *M* iodine, and secondly the less satisfactory addition of excess $FeCl_3$ solution and back-titration with titanous chloride. The 0·0044 *M* solutions were oxidised by agitation by a stream of air and estimated iodimetrically. The data are not incompatible with the gasometric data now presented. In fact, the seemingly erratic behaviour and inconsequent relation to p_H that was then confusing is, between p_H 2 and 5, now found to be characteristic. Under both conditions of measurement, the reaction is found to have a linear velocity constant.

It is interesting finally to note that a stream of nitrogen produced, during a period of 45 min. at p_H 2·5, no change in the dialuric acid titre.

Effect of temperature.

The effect of temperature, as indicated by several experiments at 25° and 30° respectively, may be defined by the relation:

$$\log k_{t_2} = \log k_{t_1} + 0{\cdot}027\,(t_2 - t_1).$$

Fig. 6 (A) portrays the variation of the velocity of oxidation of 0·0066 *M* dialuric acid with temperature. The full curve is the curve at this concen-

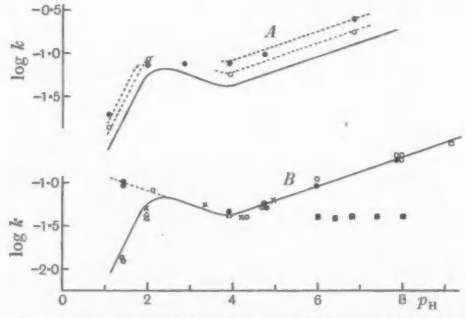


Fig. 6. A. Effect of temperature. ○ 25°, • 30°. B. Effect of added catalysts, ○ cyanide, • iron (data of Hill ■), □ glass rods, × barbituric acid, △ oxidised solution. log k: p_H curve (18°) from Fig. 5. 1·2 cc. 0·0066 *M*.

tration from Fig. 5, and the broken curves are the calculated curves for 25° and 30°, assuming the above relation to hold throughout the p_H range. It is noticeable that, whereas the p_H ranges 1 to 2 and 2 to 11 are separately differentiated with regard to catalysis or change of concentration, both p_H ranges are satisfactorily described by the one temperature coefficient.

Catalytic agents.

The roughly linear progress of oxidation is general evidence of a catalytic reaction embodying a constant concentration of catalyst. Further light upon the nature of this catalysis has been sought. In the first place, experiments were conducted at similar p_H values with different buffer solutions. Dilute HCl was used for the range p_H 0·7 to 1·7, citrate buffer was used for p_H 1·4 to 5, and universal buffer for p_H 2 to 11. No consistent differentiation was apparent, and no distinction is therefore made in Fig. 5 as to the buffers used in the respective experiments.

Although this in itself is probably sufficient evidence that catalysis was not due to the fortuitous presence of iron, definite observations on the catalytic effect of Fe and KCN on the oxidation are presented in Fig. 7 (A). It is clear

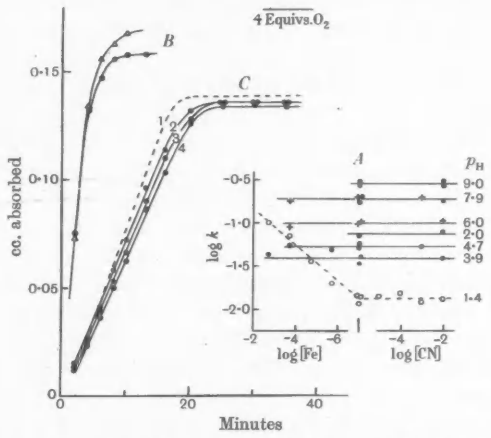
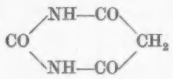


Fig. 7. A. Effect of iron and cyanide ions. • o not CO_2-free, + CO_2-free. B. Effect of glass surface. p_H 7·9. • plain, △ glass rods. C. Effect of constituent impurities. p_H 2·0. 1 plain, 2, 3 and 4, 0·005, 0·004 and 0·0016 *M* added oxidised solution, respectively. 1·2 cc. 0·0066 *M*.

from this that there is, except in acid solution, no catalytic effect of iron (or inhibitory effect of KCN) upon the reaction. This finding is especially noteworthy since the formation of the well-known blue iron complex in slightly alkaline solution (0·0016 *M* Fe, p_H 7·9) is thus found to be entirely without catalytic influence. In the case of acid solution, addition of cyanide was found to have no effect, whereas addition of iron led to steadily increasing velocities, defined in Fig. 7 (A) by the arbitrary straight line, $k = \sqrt[5]{[Fe]}$.

The total oxygen absorptions in the presence of smaller concentrations than 0·0001 *M* Fe or 0·001 *M* KCN, respectively, were of the usual extent. In the presence of 0·00016 *M* iron, however, oxygen absorptions were less than this, and with 0·0016 *M* iron were quite definitely one-half the usual extent even assuming partial oxidation by added iron (hence $n = 0{\cdot}5$). In alkaline Fe solution a certain confusion appears to arise from liberation of CO_2. Conversely, in the presence of 0·0083 *M* KCN, at p_H above 6, absorptions up to double the usual extent ($n = 2$) were obtained, although normal uptakes occurred at p_H values acid to 6.

Since the identity of velocity in different buffer preparations suggested the absence of catalytic impurity from the buffer, the dialuric acid preparation was next examined. The possibility of catalysis by a pyrimidine of similar structure was tested by the addition of barbituric acid,



possibly a contaminant by excessive reduction during preparation. No effect was observed, nor was barbituric acid itself oxidised. Next, a 0·02 *M* solution of dialuric acid in 0·001 *M* HCl was completely oxidised in a Warburg flask, and added, in final concentration 0·005 *M*, 0·004 *M*, and 0·0016 *M*, to fresh 0·0066 *M* dialuric acid solutions for oxidation experiments. Although the presumed catalyst must have been present in increased concentration at the initiation of these oxidations, the curves of Fig. 7 (C) show perfectly clearly that there has been no acceleration of the reaction.

The final possibility that catalysis was a surface phenomenon due to the glass container was tested by adding fine glass rods to increase the surface area of glass. Although the increase in wetted surface was from about 15 to about 30 cm.², no increased velocity was measured (Fig. 7 (B)).

In Fig. 6 (B), values of $\log k$ under different "catalytic" conditions in the oxidation of 0·0066 *M* dialuric acid are assembled, and compared with the mean curve for this concentration transcribed from Fig. 5.

The notable absence of catalysis induced extensively by any of the adopted procedures makes the linear order of reaction difficult to explain on any assumption that does not consider as a limiting factor the rate of permeation or of activation of oxygen. The fact that the one effective catalysis observed (Fe at p_H 1·3) has increased $\log k$ just so far as to align it with its more alkaline values while these values cannot themselves be increased, is itself rather significant in this respect. It appears that values greater than this cannot be obtained by catalytic acceleration of the oxidative reaction.

Effect of concentration.

A final indication as to the nature of the catalysis should be procurable from the relation of k to m. If z be g.-mol. of dialuric acid oxidised in time t, then when the reaction is limited by constant extraneous factors, such as

rate of activation of oxygen or catalysis by a constant impurity of the glass or buffer, the molar velocity constant should be $= z/t$. On the other hand, if the extraneous factors vary with m (*e.g.* catalysis by a constituent of the dialuric acid solution), the constant should be z/mt; this situation, however, has been rendered very improbable by Fig. 7 (C). Now k has actually been calculated as $x/t\,(= z/mt)$. k is therefore likely to vary, in accordance with the first situation, as $1/m$.

In Fig. 8, the comparison of velocity with concentration of dialuric acid is made by transcribing the curves of Fig. 5 upon a common ordinate. It is clear that at no p_H does $\log k$ vary as $-\log m$ in accordance with the requirements for constant catalytic activation. Indeed, between p_H 1 and 2, $\log k$ is very approximately non-variant with m, whereas on the alkaline side of p_H 4, it corresponds notably with the unforeseen relationship, $k = C''\,(1/\sqrt{m})$, indicated by the broken lines in Fig. 8.

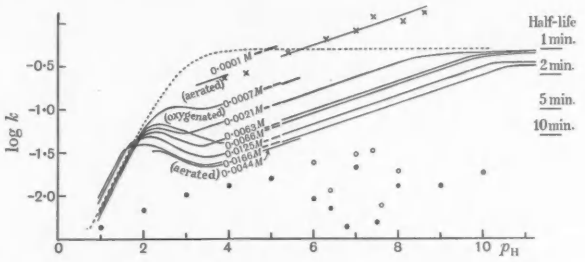


Fig. 8. Variation of $\log k$ with concentration of dialuric acid. •○ Data of Hill (0·018 and 0·0062 *M*, respectively). × Data of Clark, Cohen and Gibbs for leucomethylene blue.

It is interesting to observe the excellent agreement of the gasometric data with the data from titration of fully oxygenated mixtures. At the same time, however, the data obtained by titration of aerated solutions are definitely low, being included merely to show a general analogy between the curves. Yet it is a pointer to an unexpected correlation with tentative observations of Clark, Cohen and Gibbs [1925] on the autoxidation of leucomethylene blue by aeration; but even though their data as presented in Fig. 8 are convincingly close to the square root relation, it would be premature to press the analogy.

The data of Hill, replotted and recalculated according to the conventions of this paper, are included in Fig. 8 for reference. As is to be expected, the velocities measured fell considerably short of the velocities determined in the present work.

Of further interest in Fig. 8 is the possibility that the decline of the alkaline points from the $\sqrt[6]{[H^+]}$ relationship may be conditioned more by the acid dissociation of dialuric acid [Richardson and Cannan, 1929] than by the technical difficulty of measurement. The dotted line represents the maximum attainable velocities on the assumption that velocity varies directly as the

concentration of the ion dial.$^-$ and is definable by the actual measurements between p_H 1 and 2. In regions not greatly alkaline, however, it is presumed that this maximum velocity is not attained owing to its limitation by the external situation already mentioned. Although the half-life of dialuric acid is, as Fig. 8 indicates, so brief that uncertainty of measurement precludes any positive conclusion, it is at least clear that this interpretation would align itself remarkably with the tendency towards constant oxidation velocities in the more alkaline regions.

The autoxidation of alloxantin.

The experiments in which oxidised dialuric acid was added to solutions awaiting measurement (Fig. 7 (C)) were analogous, in effect, to measuring the autoxidation of alloxantin. Nevertheless, distinctive reaction velocities in these cases were not obtained. In seeking to confirm the observation, reactions were now conducted with freshly prepared alloxantin solutions, using an identical technique with that for dialuric acid. Owing, however, to the limited solubility of alloxantin, reaction mixtures more concentrated than 0·0055 *M* could not (with the exception of measurements in HCl) be obtained. Needless to say, alloxantin solutions of this strength remain only slightly associated, and are actually equivalent, as calculated from the association constant, to 0·0050 *M* solutions of dialuric acid. Fig. 9 depicts the results in the usual

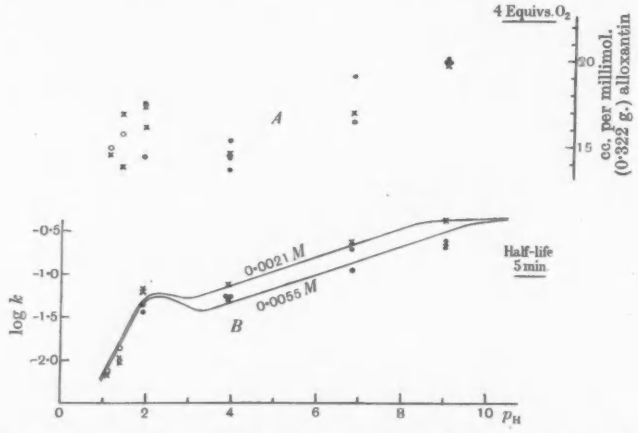


Fig. 9. Autoxidation of alloxantin. A. Total absorption. B. log *k*. 18°. ○ 0·0067*M*, • 0·0055*M*, × 0·0021*M* alloxantin.

fashion upon a basis of curves already defined, where, however, the curve for 0·0055 *M* solutions is calculated from the observed relation $k = C\,(1/\sqrt{m})$ (m = 0·005 *M* dialuric acid). The results clearly differ in no essential respect from those for dialuric acid. The oxygen uptakes were also of similar magnitude,

a selection of data for the more concentrated solutions being depicted in Fig. 9 upon a separate ordinate. Thunberg [1915], in two experiments on unbuffered saturated alloxantin solutions, measured uptakes in both cases in excess of two equivalents of oxygen, at rates such that the half-life of alloxantin was about 4 to 5 mins.

Alternative velocity constants.

The very striking variation of k as $1/\sqrt{m}$ over the p_H range 4 to 11 (Fig. 8) made it desirable to investigate the velocity equation

$$\frac{dz}{dt} = k_s' \sqrt{m - z},$$

from which by integration

$$k_s' = \frac{2\sqrt{m}}{t}\left(1 - \sqrt{1 - \frac{z}{m}}\right).$$

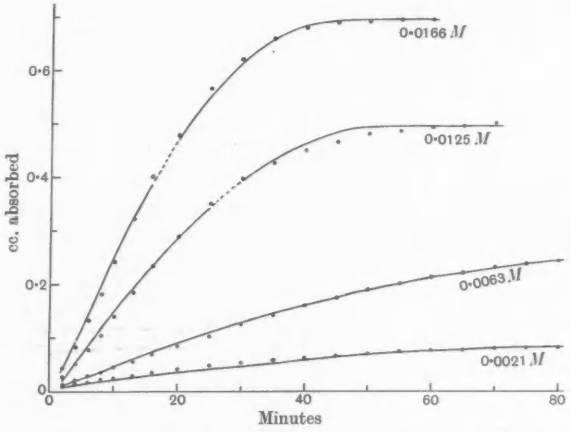


Fig. 10. Alternative velocity curves. 2·4 cc. p_H 1·4. HCl buffer. $\frac{dx}{dt} = k_s\sqrt{1-x}$.

Using x instead of z, these relations reduce to

$$\frac{dx}{dt} = k_s\sqrt{1-x} \text{ and } k_s = \frac{2}{t}(1 - \sqrt{1-x}),$$

in which k_s is non-variant with m. Fig. 10 shows that the expression fits the data for the slower and better defined oxidations with a fair degree of precision. Since it may be also calculated that

$$k = k_s\left(1 - \frac{k_s t}{4}\right),$$

or, provided the constants are calculated from early points such that kt is small compared with 4, that

$$k = k_s = \frac{k_s'}{\sqrt{m}},$$

variation of k_s with p_H is already satisfactorily defined in Fig. 5. These relations of k_s would derive chemically from the not impossible assumption that the

reaction is catalysed by both charges of the ion dial.$^{--}$, presumably by way of a double peroxide from two molecules of added oxygen.

Yet the formulation provides no better explanation of the region p_H 4 to 11 than does the simple linear reaction. In both cases, limitation of the velocity below the maximum formulated rates appears to operate. Again, whereas both velocity constants theoretically remain non-variant with concentration, the variation actually is as $1/\sqrt{m}$ in either case. In general, indeed, the relation

$$\frac{dx}{dt} = k_n (1 - x)^n$$

shows that k_n, for any order of reaction calculated in terms of x (which is non-variant with m), should itself be non-variant with m. Consequently, for the region p_H 4 to 11, where explanation in terms of linear constants has already been found inadequate, no explanation in terms of the ordinary velocity constants can be provided.

Discussion.

The fact that at p_H 7 oxidation was complete in considerably less than 10 mins. means that the experiments of Hill [1930; 1931] were conducted on solutions already considerably oxidised. This was initially suspected in a technique which exposed neutral solutions of dialuric acid to the atmosphere for an undetermined time before measurement, and which yielded thereafter plainly erratic total absorptions of oxygen. It was, however, an unfortunate circumstance that the few duplicates described in the second paper gave a general confirmation of the unduplicated findings of the first. In both cases, nevertheless, absorptions appear to have been very considerably below the maximum simply by reason of this initial oxidation (*cf.* Fig. 2).

It cannot be held therefore that alloxantin is the end-product of the reaction (an event unlikely by reason both of the large dissociation to its constituents in dilute solution, and of the existing evidence of Thunberg), since autoxidation has now been shown to proceed to completeness in definite stoichiometric proportions. The velocity equations of Hill are therefore in error. Again, for the reasons that the volume of solution was too large for efficient aeration (10 cc. in 33 cc. flasks), and that oxidation was in any case already far advanced, the actual velocities measured do not represent oxidation of dialuric acid under reproducible conditions, and can have no final significance (*cf.* Fig. 8). It is thus impossible to continue using this evidence to relate the oxidation-reduction in any way with the metabolic oxidation of cell material.

On the other hand, the oxidation of amino-acids in the presence of dialuric acid described in Hill's third paper [1932] is of considerable interest. But whether this is due merely to products of autoxidation, or to a definite reversible oxidation-reduction of dialuric acid has not yet been sufficiently defined. H_2O_2 is known to oxidise amino-acids in the given manner [Negelein,

1923; Breinl and Baudisch, 1907], while Hill discusses the similar reactions of alloxan. Unfortunately, however, the stoichiometry of either reaction near p_H 7 is not known. But it is conceivable that the end-products of oxidation of each equivalent of dialuric acid, may oxidise of themselves at least two equivalents of amino-acid. Therefore, since Hill measured greater values than this in only 2 of 11 experiments in reactions exceeding 5 hours, his evidence for catalysis by repeated oxidation-reduction of dialuric acid, remains inconclusive. In any event, it should be noted that alloxan as an individual constituent of cell material would have a half-life period not exceeding 2 mins. at any cell p_H alkaline to 6·5[1].

The possibility that both electrode potential and velocity of oxidation vary as a function of the dissociation constant of dialuric acid has been neither discounted nor more definitely manifested by the results of these experiments. But general correlation between potential and velocity has not been attained. The nature of the reaction appears to have been obscured by extraneous factors which the rapidity of oxidation allows to become operative.

Before considering these effects therefore the limitations of the technique should be briefly examined. Gasometric experiments have been conducted at 18°, the lowest temperature consistently available, and with air instead of oxygen as used previously, to minimise the rate of oxygen absorption. Nevertheless, the average absorption was at the rate of 1·5 to 3·0 cc. per hour, with a maximum of 12 to 15 cc. per hour. Dixon and Elliott [1930] state that under their conditions (32 cc. gas space, paraffin manometer fluid) measurements at rates limited to 1 cc. per hour are perfectly reliable, and they measure rates of 2·4 and 3·0 cc. per hour for cysteine and yeast without difficulty. Indeed, for rates of oxidation of pyrogallol of 18, 26 and 31 cc. per hour, only in the last case was there clearly discernible inconsistency. It is therefore probable that the rates measured herein are not greatly in error technically. Of this, the satisfactory concord between gasometric and titrimetric data is added evidence. This concordance of data is also evidence that aeration under the optimum conditions defined in Fig. 3 was actually equivalent to the complete oxygen saturation employed for the titrimetric data.

The extent of the error thought by Dixon and Elliott to arise from autoxidation of filter-paper under 40 % NaOH, does not appear to have been large under the present conditions, since no great discrepancy between oxygen absorption in the presence and absence of filter-paper-NaOH appears in Fig. 2.

As to stoichiometry, there is no doubt from Fig. 2 that, whereas only two electron equivalents are necessary for oxidation to alloxan, actually four equivalents of oxygen have reacted. Presumably this reaction is represented by: dialuric acid + O_2 = alloxan + H_2O_2. In the presence of iron, however, only two equivalents were absorbed; but since such conditions imply that H_2O_2

[1] Fig. 2, Section A of the previous publication [Richardson and Cannan, 1929] portrays, as is clear from Table V, the variation of log $10k$ with p_H, not of log k as indicated.

is not likely to be a stable end-product owing to its enhanced oxidising activity the reaction may be expected to proceed with one-half the oxygen absorption and no production of H_2O_2. It is interesting that the iodine titre of dialuric acid after oxygen absorption at p_H 5, 9 or 10 and acidification had entirely vanished. In such conditions, iodine does not react spontaneously with H_2O_2 [Liebhafsky, 1931].

The actual linear progress of the reaction characteristic between p_H 2 and 11 (*cf.* Figs. 2 and 4) is difficult to interpret. Not only did it occur where air-saturation was maintained by agitation, but even under conditions where the solution was pre-saturated with oxygen. It is therefore not a function of the rate of permeation of oxygen. Yet no change in the catalytic environment was able to point to its catalytic causation. It seems probable that the reaction is some obscure function of the rate of activation of oxygen, a conclusion supported by the positive temperature coefficient of the reaction, and by the unusual relations with $[H^+]$ and m. These relations may be defined by the equation $k' = k\sqrt{m}\sqrt[6]{[H^+]}$ or $k\sqrt{m}\sqrt[5]{[H^+]}$, where k' is a universal constant for the reaction between p_H 4 and 11. It is appropriate to recall that the tentative observations of Clark, Cohen and Gibbs [1925] described k for the autoxidation of leucomethylene blue also as a linear constant which varied as $\sqrt[5]{[H^+]}$, and that the magnitude of k is found in Fig. 8 to be very little removed from the $\sqrt{m}$ relationship described for dialuric acid.

Between p_H 1 and 2, the progress of oxidation is not so definitely linear, and may be described by an alternative velocity equation. k, by either derivation, is found to be non-variant with m and to vary inversely as $[H^+]$, relations which would follow directly from the chemical assumption that dialuric acid is oxidisable only in the form of its ion. It is interesting that addition of iron, which had no effect alkaline to p_H 2, between p_H 1 and 2 causes the reaction to become definitely linear and to proceed at velocities similar to those in more alkaline solution.

It is therefore suggested that in the autoxidation of dialuric acid under the observed conditions, the rate of activation of the reactants is of finite magnitude and varies between p_H 4 and 11 as $\frac{1}{\sqrt{m}\sqrt[5]{[H+]}}$; further, that where, by reason of p_H or of iron catalysis, the rate of reaction is greater than the rate of activation, the observed oxygen uptakes are conditioned in speed by the rate of activation. The velocity of the true reaction is therefore only observed where oxygen uptake proceeds slowly, a condition which is not fulfilled except between p_H 1 and 2.

Summary.

The rate of atmospheric oxidation of dialuric acid has been studied with reference to p_H, temperature, concentration of dialuric acid and influence of catalysts.

The reactants have been shown to combine in equimolecular proportions. In the presence of iron as catalyst, the proportion of oxygen was halved.

Alloxantin behaves as though completely dissociated to its components, dialuric acid and alloxan.

The suggestion that the oxidation-reduction has an important rôle in biological respiration has been rejected.

The author wishes to thank Prof. R. K. Cannan for his continued interest and encouragement during the course of this work.

REFERENCES.

Biilmann and Bentzon (1918). *Ber. deutsch. chem. Ges.* **51**, 522.
Breinl and Baudisch (1907). *Z. physiol. Chem.* **52**, 167.
Clark, Cohen and Gibbs (1925). *U.S. Public Health Reports*, **40**, No. 23, 1131.
Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Hill (1930). *J. Biol. Chem.* **85**, 713.
—— (1931). *J. Biol. Chem.* **92**, 471.
—— (1932). *J. Biol. Chem.* **95**, 197.
Liebhafsky (1931). *J. Amer. Chem. Soc.* **53**, 2074.
Negelein (1923). *Biochem. Z.* **142**, 493.
Randall and Failey (1927). *Chem. Rev.* **4**, 271.
Richardson and Cannan (1929). *Biochem. J.* **23**, 68.
Thunberg (1915). *Skand. Arch. Physiol.* **33**, 217.

APPENDIX.

A selection of the data from which k has been calculated for 0·0166 M solutions is given below:

2·4 cc.; 18°.

p_H	Buffer	y	mm.³/min.	Total uptake (mm.³)	p_H	Buffer	y	mm.³/min.	Total uptake (mm.³)
1·1	HCl	0·97	12	625	4·8	Citr.	0·98	31	560
1·4	,,	0·95	27	695	6·0	U.B.	1·00	50	705
1·4	Citr.	0·95	29	660			0·96	54	735
2·0	U.B.	0·99	32	700	6·8	,,	0·98	59	760
2·5	Citr.	0·95	28	640			0·97	62	745
		0·99	27	650	7·9	,,	0·97	76	780
2·9	U.B.	0·97	21	590			0·97	90	775
3·3	Citr.	0·99	22	500			1·00	130	730
		0·99	26	550	9·0	,,	0·97	150	780
3·9	U.B.	0·97	24	600			1·00	180	740
		0·96	23	505	9·7	,,	0·99	220	765
4·7	,,	0·98	32	625	11·0	,,	0·99	275	755

CCXXXIV. FAT METABOLISM IN FISHES.

I. GENERAL SURVEY OF THE FATTY ACID COMPOSITION OF THE FATS OF A NUMBER OF FISHES, BOTH MARINE AND FRESH-WATER.

By JOHN ARNOLD LOVERN.

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research.

(*Received October 20th, 1932.*)

The problems of fat metabolism in fishes appear in several ways to be somewhat different from those met with in the study of fat metabolism in either plants or mammals. The mixtures of fatty acids which go to form the fat of plants or mammals are much simpler than those of fishes, and it is difficult to find a reason for the special compositions of fish fats, with their numerous unsaturated components and high molecular weights. Two factors are probably of importance.

(*a*) Cold-blooded animals in general lay down a more unsaturated type of fat than warm-blooded ones. The effects of temperature on degree of unsaturation, as affecting both plant and animal depôt fats, are well established. This, however, will not explain the presence of fatty acids of such high molecular weight. It also does not account for the fact that the fats of marine mammals are mostly similar in general composition to those of the cold-blooded fishes.

Of the common marine species most are exclusively carnivorous, eating other fish. Thus their own fat composition will be decided largely by the type of fat in their food, since their carbohydrate intake is low, and fat synthesis from this source is not likely to be great. True, since protein can be transformed into carbohydrate, there is a possibility of fat being formed from protein, but the process seems an extravagant and wasteful one, and the writer is unaware of any evidence in support of this hypothesis. Thus it seems evident that if the smallest marine animals, which feed on marine flora, lay down these special fats, they will automatically bring about the storage of closely allied fats in the larger fish. It is therefore of interest to note that the marine flora contains poly-unsaturated fatty acids of high molecular weight similar to those found in the fat of fishes, although in very small amounts [Tsujimoto, 1925].

The number of species of fish whose fat has so far been quantitatively investigated is small, and it is to be noted that no single fresh-water species is

included. This is significant in view of the possibly different food supply of most fresh-water fish.

In 1930, Guha *et al.* [1930] published a paper in which was given a list of the quantitative compositions of the fatty acids of all the fish fats examined up to that date. This included only 13 species, and the authors pointed out that it was important that their survey should be greatly extended, in order to substantiate the biological group classifications which they made. This paper was followed by one from the writer [Lovern, 1930], in which further support was given to the previous authors' suggestions as to the peculiar relationships holding in the Elasmobranch family. Since that date the writer has not found opportunity to pursue this particular point further, but it is certainly one which will require a more detailed consideration. The only other quantitative data which the writer has observed are the partially quantitative analysis by Tsujimoto [1932] of a shark-liver oil of low iodine value, and the earlier work of Gill and Tucker [1930] on the composition of porpoise-jaw oil. The existence of such an oil as the last mentioned, of which the characteristic acid is *iso*valeric, illustrates one of the extraordinary complexities of fat metabolism in fishes.

The methods employed in the present work were practically identical with those given in detail in earlier papers [Guha *et al.*, 1930] and will not be described here. Considerations of space also preclude the giving of all the analytical data in detail, and only the final compositions will be included. One slight modification of method deserves mention. In the process of separating into liquid and solid acids by lead salt/alcohol, Banks [1932] found a much more complete separation when 0·5 % of acetic acid was added to the alcohol in the first precipitation. (This is, of course, always added of necessity in the subsequent recrystallisation of the solid lead salts.) The writer has used this new process with entire success.

In Table I are given the particulars of the oils, and in Tables II and III the percentage compositions of the fatty acid mixtures present in the new marine species examined. Table II is expressed in weight percentages, since this is the way in which previously recorded compositions have invariably been given, and Table III is expressed in molar percentages. In Table III is included an average of the previous group classifications, brought to molar percentages. The advantage of considering results from a molecular standpoint,

Table I. *Particulars of marine oils examined.*

Species	Description of oil
Haddock (*Gadus aeglefinus*)	Oil from the liver, the main storage depôt Iodine value 168·5 and unsaponifiable matter 0·7 %
Sprat (*Clupea sprattus*)	Oil from the head and body tissues, the main storage depôt Iodine value 150·7 and unsaponifiable matter 0·9 %
Angler (Monk) fish (*Lophius piscatorius*)	Oil from the liver, the main storage depôt Iodine value 162·3 and unsaponifiable matter 1·0 %
Halibut (*Hippoglossus vulgaris*)	Oil from the liver, one storage depôt Iodine value 120·0 and unsaponifiable matter 6·6 %

Table II. *Composition of mixed fatty acids (weight percentages) of oils of marine species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Haddock	4·3	14·1	0·3	0·5	12·4	30·5 (−2·6 H)	29·3 (−5·9 H)	8·6 (−7·3 H)
Sprat	6·0	18·7	0·9	0·1	16·2	29·0 (−2·9 H)	18·2 (−5·6 H)	10·9 (−7·1 H)
Angler fish	4·9	9·6	1·3	0·4	12·1	30·9 (−3·3 H)	24·9 (−5·9 H)	15·9 (−8·6 H)
Halibut	3·9	15·1	0·5	Nil	18·7	34·4 (−2·0 H)	13·8 (−5·5 H)	13·6 (−7·6 H)

Table III. *Composition of mixed fatty acids (molar percentages) of oils of marine species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Haddock	5·2	15·4	0·3	0·6	13·6	30·5 (−2·6 H)	27·1 (−5·9 H)	7·3 (−7·3 H)
Sprat	7·2	20·1	0·9	0·1	17·6	28·5 (−2·9 H)	16·5 (−5·6 H)	9·1 (−7·1 H)
Angler fish	6·0	10·6	1·3	0·5	13·5	31·2 (−3·3 H)	23·2 (−5·9 H)	13·7 (−8·6 H)
Halibut	4·7	16·3	0·5	Nil	20·4	34·0 (−2·0 H)	12·6 (−5·5 H)	11·5 (−7·6 H)
Gadidae*	6·5	12·0	Trace	Trace	15·5	29·0	25·5	11·0
Clupidae*	7	14·0	1·5	Nil	15·5	27·0	21·0	13·0
Balaenidae*	7·5	11·5	3·0	1·0	18·0	38·5	Variable	10·5

* Summarised from work of Guha *et al.* [1930]. Figures for Elasmobranch group, and sperm whale oils not included.

rather than one of mass has been pointed out by Hilditch and Sleightholme [1930].

The average unsaturation of the various groups has been expressed in terms of the number of hydrogen atoms required for saturation, as in previous papers. These values are in agreement with those previously recorded [Guha *et al.*, 1930] for the Gadidae and Clupidae oils. It is to be noted that each oil was a representative sample from a large number of fish, but even so, seasonal and other changes will account for minor fluctuations. In general, it will be seen that the oil from haddock-liver falls quite well into the Gadidae group, and that of the sprat into the Clupidae. These two groups are similar in composition. Angler fish-liver oil is similar to both. Halibut, however, is peculiar in that linoleic acid appears to be absent, and the proportions of acids of the C_{20} and C_{22} groups are nearly the same. Normally the C_{20} group greatly predominates. This seeming lack of linoleic acid will be referred to again. Conjointly with the reduced amount of acids of the C_{20} group it will account for the low iodine value of halibut-liver oil.

Up to the present five oils from fresh-water fish (4 species) have been examined in detail, and in Tables IV, V and VI are given the particulars and the compositions.

Table IV. *Particulars of oils from fresh-water fish.*

Species	Description of oil
Carp (*Cyprinus carpio*)	Oil from head and body tissues, the main fat depôt Iodine value of oil = 122·9 and unsaponifiable matter = 3·5 %
Pike (*Esox lucius*)	(*a*) Oil from head and body tissues, one of two main fat stores Iodine value of oil = 145·9 and unsaponifiable matter = 4·0 % (*b*) Oil from the mesentery, the other main fat depôt Iodine value of oil = 134·2 and unsaponifiable matter = 0·1 %
Pollan (*Coregonus pollan*)	Oil from head and body tissues, the main fat depôt Iodine value of oil = 136·8 and unsaponifiable matter = 1·5 %
Perch (*Perca fluviatilis*)	Oil from head and body tissues, the main fat depôt Iodine value of oil = 131·7 and unsaponifiable matter = 6·0 %

Table V. *Composition of mixed fatty acids (weight percentages) of oils of fresh-water species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Carp	3·7	14·6	1·9	1·0	17·8	45·8 (−3·2 H)	15·2 (−6·9 H)	Nil
Pike (body)	4·7	13·2	0·5	0·8	20·8	38·4 (−2·8 H)	15·3 (−7·5 H)	6·3 (−7·5 H)
Pike (mesentery)	2·9	15·0	Trace	0·5	20·2	42·4 (−3·4 H)	15·1 (−6·7 H)	3·9 (−8·0 H)
Pollan	2·9	14·3	1·9	1·5	19·8	40·0 (−3·2 H)	13·5 (−7·4 H)	6·1 (−9·1 H)
Perch	3·5	12·5	2·0	1·1	19·4	40·5 (−3·2 H)	13·8 (−6·8 H)	7·1 (−9·2 H)

Table VI. *Composition of mixed fatty acids (molar percentages) of oils of fresh-water species.*

	Saturated			Unsaturated				
Species	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Carp	4·4	15·5	1·8	1·2	19·0	44·5 (−3·2 H)	13·6 (−6·9 H)	Nil
Pike (body)	5·6	14·1	0·5	1·0	22·4	37·4 (−3·0 H)	13·8 (−7·5 H)	5·2 (−7·5 H)
Pike (mesentery)	3·4	16·0	Trace	0·6	21·7	41·5 (−3·4 H)	13·6 (−6·7 H)	3·2 (−8·0 H)
Pollan	3·4	15·5	1·8	1·8	21·2	39·0 (−3·2 H)	12·2 (−7·4 H)	5·1 (−9·1 H)
Perch	4·2	13·4	1·9	1·3	20·9	39·8 (−3·2 H)	12·5 (−6·8 H)	6·0 (−9·2 H)

Comparing these data with those for the marine oils (Table III), we find:

(*a*) The oleic-linoleic acid group percentage consistently higher in fresh-water fish fats.

(*b*) The C_{20} and C_{22} groups present in considerably smaller amounts in fresh-water fish fats.

(*c*) Palmitoleic acid is a more important component of all the fresh-water fats than of the marine ones, with the exception of halibut-liver oil, which is unique.

(*d*) Otherwise, the fresh-water and marine oils are similar, in that in each class the average unsaturation of the various groups is of the same order, and the general proportions of the fatty acid mixture are the same, *viz.* small quantities of myristic acid, with traces only of myristoleic acid; considerably greater quantities of palmitic and palmitoleic acids (in comparable amounts); traces only of stearic acid, with oleic and linoleic acids as one of the main components of the oils; the C_{20} group, having no saturated member, also a major constituent, and the C_{22} group, still more unsaturated, in smaller amount.

Banks and Hilditch [1932], working on the body fats of the pig, have shown that there is a balance between stearic and oleic acids, tending to keep the total amount of C_{18} acid approximately constant, however much either component varies. Stearic acid is not a major component of fish oils, but palmitic is, and it is of interest to compare the total group percentages (molar) for all the oils described.

Table VII. *Total groups of acids. Molar percentages.*

Species	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Marine:					
Haddock	5·8	29·0	30·8	27·1	7·3
Sprat	7·3	37·7	29·4	16·5	9·1
Angler fish	6·5	24·1	32·5	23·2	13·7
Halibut	4·7	36·7	34·5	12·6	11·5
Gadidae (average)	6·5	27·5	29·0	25·5	11·0
Clupidae (average)	6·0	27·2	28·7	22·5	15·5
Fresh-water:					
Carp	5·6	34·5	46·3	13·6	Nil
Pike (body)	6·6	36·5	37·9	13·8	5·2
Pike (mesentery)	4·0	37·7	41·5	13·6	3·2
Pollan	5·2	36·7	40·8	12·2	5·1
Perch	5·5	34·5	41·7	12·5	6·0

It will be noticed that there is a tendency towards an approximate constancy of the amount of C_{16} acids in the fresh-water fish, but no constancy in the marine species. This may, however, be fortuitous with so few samples. It may be significant that the two marine fats with total C_{16} acids of 37 % (sprat and halibut) are also comparable with the fresh-water fats in having a low C_{20} content.

In the paper of Guha *et al.* [1930] it is stated that the poly-unsaturated acids of high molecular weight characteristic of fish oils are evidently marine in origin. This cannot now be accepted unreservedly—they appear to be characteristic of aquatic life (including marine algae, *etc.*) of all kinds, including fresh-water. The diet of fresh-water fish is perhaps different from that of marine species. Certainly many are purely carnivorous, but in many cases they eat such species as the young of small birds (the particular pike examined,

for instance, all had small wild-ducklings in their stomachs), frogs, and even mice, *etc.* Others are herbivorous. It has not been shown whether the small crustacea, worms, *etc.*, and the flora of fresh-water contain these typical fish oil acids. It is hoped later to investigate this point.

The high content of oleic and linoleic acids in the fats from the fresh-water fish is paralleled in the marine mammalia (Balaenidae), but the parallelism does not extend to the other groups of acids. However, in all land mammals, the oleic-linoleic acid group is the only unsaturated one present, and hence we may say that the fats from fresh-water fish, as well as those from marine mammals, are of a type intermediate between the fats from marine and terrestrial animals. Differences of food might account for this, if the natural tendency of all fish was to lay down fats of the marine type. It is hoped later to conduct controlled feeding experiments on both fresh-water and marine species to decide this point, but the technical difficulties are considerable. Two other factors must be borne in mind. The average temperature of fresh-water ponds and rivers, especially shallow ones, is likely to be higher than that at the bottom of the sea. Also, the salinity of sea-water may have some effect, although the writer does not consider this probable. It can fairly easily be determined with species which can be kept in both fresh and salt water, *e.g.* eels. It is well not to underestimate the effects of environmental and seasonal differences, as contrasted with the effects of food differences, since the work of Hilditch and Sleightholme [1930] has shown that, in the case of butter fats at all events, the former may far outweigh the latter.

The apparent lack of linoleic acid in the case of halibut-liver oil requires further notice. It is now almost certain that mammals, for example, cannot synthesise linoleic or linolenic acids from carbohydrate, and that when deprived of them in their diet they suffer in health. Administration of either acid will alleviate the trouble [Evans and Lepkovsky, 1932; Burr, Burr and Miller, 1932]. Earlier work on different lines by Ellis and Harkins [1925] on the composition of pig fats lends decided support to this view, that all the linoleic acid present has come directly from ingested linoleic acid. The results of Hilditch and his collaborators [Banks and Hilditch, 1932; Bhattacharya and Hilditch, 1931] on pig fats are also in accordance with this theory. There is no evidence as to whether or not linoleic acid is essential to the well-being of fishes, but it is significant to note that the highly unsaturated arachidonic acid did not alleviate the symptoms in the experiments of Burr, Burr and Miller [1932]. Either linoleic acid is not essential to the halibut (it must be remembered that the diet of the halibut will include linoleic acid), and is hence readily destroyed by oxidation, or else it was present, but in traces too small for detection by the analytical methods employed. There is a third possibility. The liver is not the only fat store in the halibut, and there are quite considerable amounts of fat deposited just beneath the skin. It would be necessary to analyse fat from every single depôt in the fish before it could be said that linoleic acid was definitely absent.

Nevertheless, the composition of halibut-liver oil is unusual in this respect, and also in its low content of acids of the C_{20} series. The writer is frequently examining samples of halibut-liver oil in connection with another piece of work, and has found that the iodine value is invariably low, and of the order of 120, as with the sample analysed. Thus the possibility of it being a "freak" sample is rather remote.

One other point is worthy of mention. It was indicated in the earlier work of Guha *et al.* [1930] that the widely varying extents to which the highly unsaturated esters tended to polymerise on heating suggested that the structures of the acids were not necessarily similar in the different oils. The writer has observed the same marked peculiarities in the present work, and also a possibly associated fact, that with some oils the highly unsaturated esters which distil over have a deep golden-yellow colour, whilst with others the distillates are almost colourless. This is quite irrespective of the iodine value of the fractions, or of the colours of the original oils, and is not due to traces of pigment. This point seems worthy of further investigation, and is being followed up.

SUMMARY.

A start has been made on a series of investigations into the peculiar problems of fat metabolism in fishes. The work of Guha *et al.* [1930] has been extended as a preliminary to more detailed work, and the present paper includes data for a number of oils, including five from fresh-water fish.

Significant differences in fatty acid composition are apparent between the oils from fresh-water fish on the one hand, and from marine species on the other. The most noticeable are the increased proportions of oleic and linoleic acids, and also of palmitoleic acid, in the fresh-water fish oils, and the reduced amounts of acids of the C_{20} and C_{22} series. Possible reasons for this are mentioned.

The composition of a sample of halibut-liver oil, representative of many fish, has been shown to be unique in respect of an apparent entire lack of linoleic acid, and of the low ratio of C_{20} to C_{22} acids. The significance of this absence of linoleic acid is discussed.

REFERENCES.

Banks (1932). Private communication.
—— and Hilditch (1932). *Biochem. J.* **26**, 298.
Bhattacharya and Hilditch (1931). *Biochem. J.* **25**, 1954.
Burr, Burr and Miller (1932). *J. Biol. Chem.* **97**, 1.
Ellis and Harkins (1925). *J. Biol. Chem.* **66**, 101.
Evans and Lepkovsky (1932). *J. Biol. Chem.* **96**, 143.
Gill and Tucker (1930). *Oil and Fat Ind.* **7**, 101.
Guha, Hilditch and Lovern (1930). *Biochem. J.* **24**, 266.
Hilditch and Sleightholme (1930). *Biochem. J.* **24**, 1098.
Lovern (1930). *Biochem. J.* **24**, 866.
Tsujimoto (1925). *Chem. Umschau.* **32**, 125.
—— (1932). *Chem. Umschau.* **39**, 50.

CCXXXV. FAT METABOLISM IN FISHES.

II. THE PERITONEAL, PANCREATIC AND LIVER FATS OF THE STURGEON (*ACIPENSER STURIO*).

By JOHN ARNOLD LOVERN.

From the Torry Research Station, Aberdeen, of the Department of Scientific and Industrial Research.

(*Received October 20th, 1932.*)

The data presented in the previous paper [1932] were all connected with fats which represented the main depôt fat of each species investigated, and, with the exception of the pike fats, the fat from only one depôt was used in each species. The quantity of any particular fat employed for a quantitative analysis of the component fatty acids should preferably be of the order of 200 g. and with less than 150 g. the accuracy will begin to fall off. This means that in many cases only the main fat depôts can be dealt with. However, with very large fish, there may be enough fat available from a number of sources. The writer was fortunate to obtain the whole of the viscera and a large piece of the peritoneal fat (the main fat store in the case of this fish) of a large sturgeon (*Acipenser sturio*) measuring over 11 ft. in length. The liver was not exceptionally rich in fat, but as it weighed 14 lbs., there was an ample supply of liver fat for analysis. The pancreas was loaded with fat, and a large sample was obtained. No opportunity was afforded for the examination of the flesh oil, but it was evident that the flesh itself was not of an oily nature, and the peritoneal fat may be taken as being the main deposit of stored fat.

The compositions of the fatty acids from these three oils are given in Tables I and II, and as before, the average unsaturation of each group is expressed in terms of deficiency in hydrogen.

Consideration of these figures reveals several interesting points. The writer was surprised to find that the liver fat was apparently no more unsaturated

Table I. *Composition of fatty acids in weight percentages.*

Oil from	Iodine value	Saturated			Unsaturated				
		C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Peritoneal cavity	126·5	7·1	14·0	0·8	0·6	23·8	35·8 (−2·9 H)	12·1 (−7·4 H)	5·8 (−8·6 H)
Pancreas	119·6	4·5	16·4	1·1	Nil	21·4	36·7 (−2·9 H)	14·5 (−6·8 H)	5·4 (−9·1 H)
Liver	125	3·0	19·2	Nil	Nil	19·5	39·6 (−2·7 H)	11·8 (−7·1 H)	6·9 (−10·0 H)

Table II. *Composition of fatty acids in molar percentages.*

Oil from	Iodine value	Saturated			Unsaturated				
		C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Peritoneal cavity	126·5	8·4	14·7	0·8	0·7	25·3	34·5 (−2·9 H)	10·8 (−7·4 H)	4·8 (−8·6 H)
Pancreas	119·6	5·3	17·4	1·1	Nil	22·9	35·7 (−2·9 H)	13·1 (−6·8 H)	4·5 (−9·1 H)
Liver	125	3·6	20·5	Nil	Nil	20·9	38·6 (−2·7 H)	10·6 (−7·1 H)	5·8 (−10·0 H)

than the main depôt fat, as indicated by the iodine values, and the more detailed analysis bears this out. This is contrary to what obtains with mammals, and from the figures given above it seems difficult to imagine a process of desaturation going on in the liver of the sturgeon. The degrees of average unsaturation of the various groups of acids in the three oils are indeed so much of the same order, that the differences might be considered as insignificant. If, however, they are taken into account, they seem to be against the theory that the liver fatty acids (it being remembered that throughout it is glyceride fatty acids that are referred to, and not phospholipin fatty acids) are more unsaturated than the fatty acids found in other fat depôts in the fish. The only grouping which is definitely more unsaturated in the liver fat than in the peritoneal fat is the C_{22} group, and for purposes of metabolism one would have thought that the acids in this group were unsaturated enough. It must not be forgotten, however, that associated with the highly unsaturated C_{22} acids there is usually some monoethylenic acid and it may be that this is further desaturated. The evidence of the other groups is against this supposition however. The C_{20} group is definitely most unsaturated of all in the storage fat, whilst the oleic-linoleic group is least unsaturated in the liver. But it is when we come to the palmitic and palmitoleic ratios that the figures seem more definite. Whilst the palmitic acid figure steadily rises from the peritoneal fat to the liver fat (*i.e.* from stored fat to fat probably taking part in active metabolism), the palmitoleic acid figure steadily falls. The total C_{16} grouping is remarkably constant, the figures being 40·0, 40·3 and 41·4 respectively for the peritoneal, pancreatic and liver fats. This steadiness between the main saturated component and its unsaturated derivative seems to the writer in full accordance with the results of Banks and Hilditch [1931; 1932] for pig fats, from which they conclude that there is inter-convertibility between saturated and unsaturated homologues. Their figures lead them to the opinion that the process is one of saturation to meet the normal body storage fat requirements, rather than one of desaturation. The present data throw no light on this interesting point, unfortunately, but they do support the argument that there is conversion of one acid into the other.

It seems of value to determine whether this relative saturation of the liver fat of the sturgeon is paralleled in the marine mammals. The writer has secured a medium-sized porpoise, and hopes to be able to pursue this point by full

analyses of the fats from various organs as well as the blubber. It must be borne in mind, of course, that fish fats are of an essentially different, and more reactive, nature than the body fats of land mammals, and their metabolism may be more direct.

There is another remarkable constancy to be observed. The total saturated acid percentages (sum of myristic, palmitic and stearic acid figures) are 23·9, 23·8 and 24·1 % respectively for the peritoneal, pancreatic and liver fats, in spite of quite considerable variations in the constituent acids—myristic from 3·6 to 8·4 %, palmitic from 14·7 to 20·5 % and stearic from 0 to 1·1 %. This strongly suggests that the three saturated acids are of equal value to the fish, and that myristic acid can replace palmitic acid functionally to a considerable extent. The lower percentage of palmitic acid would be compensated for by conversion of the excess palmitic into palmitoleic acid, thus keeping the total C_{16} acid percentage approximately constant. The net result, therefore, is strikingly in agreement with the conclusions of Banks and Hilditch, and we may picture the scheme of things in the sturgeon as follows.

The fat in all deposits requires an approximately constant relationship between the amounts of saturated and unsaturated acids present, *viz.* 24 % of saturated and 76 % of unsaturated. The exact significance of the variations in the percentages of the respective components within the saturated group is not clear, but it seems not improbable that the greater reactivity of myristic acid as compared with palmitic, and the different conditions of oxidation, *etc.*, in the various organs may have something to do with it. At any rate, we may suppose that if myristic acid is being destroyed at an excessive rate, palmitoleic acid will be saturated to increase the palmitic percentage, and so keep the ratio between saturated and unsaturated acids constant. On the other hand, we must equally suppose that if myristic acid is entering a depôt in unusually large amounts, some palmitic acid will be desaturated to palmitoleic acid. Feeding experiments will be necessary to settle this point. The rôle of stearic acid may well be similar to that of palmitic acid, but in a minor degree. The writer would have been disinclined to draw such far-reaching conclusions from the data of one fish, had not the work of Banks and Hilditch afforded such strong support.

Naturally, such relationships would not be expected to hold amongst all the oils from various marine species, as there seems no reason why all gadoid fish, for instance, should require the same ratios. It may be fortuitous that for all the fresh-water fish oils given in the previous paper, the sum of the myristic, palmitic and stearic acid percentages approximates to 20.

Two further points of interest are the following:

(*a*) In respect of the high content of palmitoleic acid, and the constant proportion of total C_{16} acids, these oils resemble those of the fresh-water fish rather than those of marine species.

(*b*) The high content of oleic and linoleic acids, together with the reduced amounts of C_{20} and C_{22} acids, is also suggestive of a fresh-water fish.

Now the sturgeon is a fish which inhabits both fresh water and salt. The particular specimen from which these fats came had been caught in the North Sea, but all sturgeon ascend the large Continental rivers to spawn, and moreover do most of their feeding in fresh water. Thus it is hardly surprising to find that the fats are of the same type as those of a true fresh-water fish, and in fact the sturgeon figures are additional support for the genuineness of the differences pointed out in the previous paper between fats from marine species on the one hand and fresh-water species on the other.

Summary.

The fats from the peritoneal cavity (main storage fat), pancreas and liver of a large sturgeon have been analysed as regards their component fatty acids. The results do not support the theory of desaturation in the liver, in the case of this particular fish. It is hoped further to investigate this point in respect of a marine mammal.

The data do, however, agree with the view that there is an inter-relationship between the main saturated acid component which has an unsaturated derivative—stearic and oleic acids in the case of pigs, *etc.*, and palmitic and palmitoleic acids in the case of fish. There is no evidence, however, as to the seat of these transformations.

There is, moreover, distinct evidence that a definite relationship is maintained between the amount of total saturated acids present, and total unsaturated acids—in the case of the sturgeon the values being 24 % of saturated and 76 % of unsaturated acids. Thus the other main saturated component, myristic acid, must enter into the scheme, and a relationship is adduced between myristic, palmitic and palmitoleic acids.

The results support in every respect the provisional conclusions drawn in the previous paper as to the characteristic differences between the fats of marine fish and fresh-water fish.

REFERENCES.

Banks and Hilditch (1931). *Biochem. J.* **25**, 1168.
—— —— (1932). *Biochem. J.* **26**, 298.
Lovern (1932). *Biochem. J.* **26**, 1978.

CCXXXVI. THE HYDROLYSIS OF CASEINOGEN BY PEPSIN AND BY TRYPSIN-KINASE.

By JOHN DEMPSTER STIRLING
and GEORGE MACFEAT WISHART.

From the Hannah Dairy Research Institute, Kirkhill, Ayr, and the Institute of Physiology, Glasgow University.

(*Received October 21st, 1932.*)

During the past few years the existence of an integral molecule of caseinogen has been questioned. Linderstrøm-Lang [1928] has extracted caseinogen with a variety of warm (60°) solvents (chiefly 60 % alcohol with or without $N/1000$ HCl and dilute HCl-NaCl solutions) and has been able to separate the protein into various fractions which differ in a number of respects, notably in their phosphorus contents. The interpretation of these experiments is difficult, for no information has, as yet, been obtained as to whether the reaction between solvent and substrate is physical or chemical. Linderstrøm-Lang, however, believes that caseinogen is heteromolecular, that it is in reality a loose physical association of several proteins of different phosphorus contents which, in all the processes to which caseinogen is ordinarily subjected, form a co-precipitation system.

Whatever interpretation be adopted, whether the various fractions are to be considered as existing chemically bound to give an integral caseinogen molecule, or whether they are held together in a species of loose physical association, it would render untenable the idea that all the phosphorus of caseinogen is concentrated in one "prosthetic" group. This is difficult to reconcile with the finding of Rimington [1927, 1] that, by the action of dilute alkali on caseinogen, he could obtain a protein containing 11 to 13 % nitrogen and only slight traces of phosphorus. Rimington [1927, 2] was also able to isolate from tryptic digests of caseinogen an enneapeptide triphosphoric ester (phosphopeptone) in amount representing about 50 % of the original caseinogen phosphorus. In terms of the Linderstrøm-Lang hypothesis, caseinogen must then be a loose physical association of large molecules, which, when subjected to the disruptive action of alkali, loses its phosphorus and still remains a co-precipitation system—the "dephosphorised caseinogen" of Rimington. Such a requirement imposes a considerable strain on the "loose association" hypothesis.

Rimington and Kay [1926] have investigated the action of pepsin and trypsin[1]

[1] Trypsin, in our paper, is used to denote the mixture of enzymes usually found in pancreatic extracts. This consists, according to the nomenclature of Waldschmidt-Leitz [1929], of trypsin (inactivated) trypsin-kinase (activated) and erepsin. Of these only trypsin-kinase has any action on caseinogen, although the others can hydrolyse its lower degradation products.

on caseinogen. They confirmed the findings of previous workers that during peptic digestion an insoluble product is formed (para- or pseudo-nuclein) which contains a large proportion of the caseinogen phosphorus. They also showed that, at the end of 9 days' digestion with pepsin, no measurable amount of free phosphate was obtained, though all the caseinogen phosphorus was now in a form not precipitated by trichloroacetic acid. With trypsin, it was found that there was a fairly rapid liberation of organically-bound acid-soluble phosphorus with accompanying slow liberation of inorganic phosphorus. The liberation of amino-acid nitrogen was followed simultaneously with that of acid-soluble phosphorus but no information was obtained regarding the partition of phosphorus between the various products formed at different stages of the digestion. Linderstrøm-Lang and his co-workers have recently [Holter, Linderstrøm-Lang and Funder, 1932] studied, in some detail, the peptic digestion of caseinogen and of the fractions obtained by Linderstrøm-Lang [1928]. They found that the precipitates (paranuclein) formed in each case had almost identical N/P ratios but that these values were quite different from that obtained by Rimington for his phosphopeptone. Linderstrøm-Lang suggests that paranuclein may be formed during tryptic digestion and that it may be the precursor of Rimington's phosphopeptone. In this connection it must be remembered that the solubility of paranuclein may be a function of p_H and its failure to precipitate during tryptic digestion may be due to the alkaline reaction of the digestion solution. None of the work quoted can therefore be taken to indicate that the action of pepsin on caseinogen is fundamentally different from that of trypsin. Waldschmidt-Leitz and Simons [1926] have demonstrated that both pepsin and trypsin-kinase act on caseinogen by the rupture of amide-linkages, *i.e.* for every amino-group set free a carboxyl is liberated. To demonstrate whether or not the same amide-linkage of caseinogen is attacked by both enzymes requires other methods of investigation than those already reviewed.

With regard to the initial attack of trypsin on caseinogen Northrop [1924] has made certain assumptions on which he has built a method for investigating the kinetics of the action of the enzyme. Northrop wished to study the effect of increase of caseinogen concentration on the rate of disappearance of caseinogen and he was forced to employ a method which, at any instant, would separate the protein from its cleavage products and so allow the amount of the former to be estimated. To this end he added trichloroacetic acid to the digestion solution at successive intervals of time and assumed that the precipitate represented unchanged caseinogen. He makes the statement that the first step in the action of trypsin, *i.e.* the hydrolysis of the caseinogen molecule itself, results in the formation of products completely soluble in trichloroacetic acid. If Northrop's conception of the initial step of the hydrolysis of caseinogen be correct, we should expect that, at any instant during digestion, the N/P ratio of a trichloroacetic acid filtrate should be identical with that of the original caseinogen. Preliminary experiments carried out by

us showed that this was by no means the case. Moreover we have found significant differences between the behaviour of pepsin and trypsin so far as the course of the acid-soluble N/P ratio is concerned, differences which appeared to indicate distinct differences in the mode of attack of the two enzymes. Accordingly, in the present paper, we report experiments designed to follow the liberation from caseinogen by the action of pepsin and of trypsin-kinase of nitrogen and phosphorus compounds soluble in trichloroacetic acid.

It would appear that no disadvantage is attached to the use of commercial pepsin preparations in view of their freedom from other proteolytic enzymes and accordingly we have employed B.D.H. pepsin (granular) in these studies. The use of commercial trypsin, however, appears to require some justification. Willstätter *et al.* [1926] have shown that, of the pancreatic proteases, only trypsin-kinase has any action on caseinogen. The others have no action on any molecule of magnitude greater than polypeptides and the latter being soluble in trichloroacetic acid the action of the peptidases will be confined entirely to the acid-soluble material resulting from the hydrolysis of caseinogen and its acid-insoluble degradation products. The change from acid-insoluble to acid-soluble will therefore be due entirely to the action of trypsin-kinase. We have used, in these experiments, an Allen and Hanbury's trypsin preparation without further purification, and we shall refer to the action of this preparation as being that of trypsin-kinase.

In view of the doubt expressed regarding the constancy of the composition of caseinogen, it is well to refer to the source of our specimen. It was prepared from milk by the improved electrodialysis method of Van Slyke [1923] and was a pure white product of N/P ratio 20·0 (caseinogen "nach Hammarsten" has N/P 18·6 while the purest specimen obtained by Van Slyke had N/P 22·2). It contained a negligible quantity of acid-soluble nitrogen and no acid-soluble phosphorus.

Experimental.

Hydrolysis by trypsin-kinase.

Caseinogen solution. This was made up fresh before each experiment and of twice the concentration desired in the digestion solution. The caseinogen was rubbed to a paste with distilled water in a mortar and dilute NaOH added slowly with stirring (0·8 cc. *N* NaOH per g. caseinogen). When all the alkali had been added the liquid was centrifuged and the clear solution removed from a slight sediment and diluted to 100 cc. with distilled water. In this way a solution of p_H slightly greater than 7·0 was obtained.

Enzyme solution. This was a 1 % filtered solution of Allen and Hanbury's trypsin and was made up fresh for each experiment. The amount of enzyme required to achieve the degree of hydrolysis desired in a given time was determined by preliminary experiment and was obtained by dilution of the 1 % solution.

Buffer solution. The optimum range of trypsin activity is p_H 7·8–8·7 [Waldschmidt-Leitz, 1923–24]. During digestion of caseinogen there is a very considerable shift of p_H towards the acid side and with the higher protein concentrations buffering is a matter of some difficulty. In our experiments the p_H has been followed throughout the course of the digestion (hydrogen electrode) and the maximum shift found was about 0·3 p_H. As the digestion solutions were always initially buffered to a p_H near to the upper limit of the optimum range it can be said that at no time, during the course of the digestion, was the enzyme subjected to a p_H below the lower limit of the optimum. The buffer used was the KCl-borate mixture (p_H 8·8) of Clark and Lubs [Clark, 1928] and was made up in a concentrated solution. (Clark and Lubs buffer was obtained by diluting 4 cc. of this concentrated solution to 10 cc.)

Procedure. A series of stoppered tubes was made up, each containing 5 cc. caseinogen solution and 4 cc. concentrated buffer solution. The tubes were placed in the thermostat and after they had attained the desired temperature (37°) 1 cc. diluted enzyme solution was added to each. At 10 or 15 minute intervals a tube was withdrawn and 10 cc. 10% trichloroacetic acid were added. After shaking, the contents were immediately filtered and acid-soluble nitrogen (micro-Kjeldahl) and phosphorus [Fiske and Subbarow, 1925] determined on aliquot portions of the filtrate. The total nitrogen and phosphorus and initial acid-soluble nitrogen and phosphorus contents were determined on enzyme-containing solutions which had not been placed in the thermostat. Controls were run by incubating tubes containing distilled water instead of enzyme solution. The changes followed by us were found to be due wholly to the presence of the enzyme as the buffer alone did not produce any hydrolysis.

Results. The amounts of acid-soluble nitrogen and phosphorus liberated at successive intervals of time with four different concentrations of caseinogen

Table I A. *Trypsin-kinase (0·5 mg. trypsin/10 cc. solution).*

Exp. No. / Fig. No.		1		2		3		4	
Total N (mg. in 10 cc. solution)		8·77		19·60		25·81		44·71	
Total P (mg. in 10 cc. solution)		0·460		0·937		1·350		2·250	
(Time mins.)		N	P	N	P	N	P	N	P
10	15*	4·66	0·334	3·08	0·384	3·43	0·203	4·41	0·25
20	30	6·50	0·421	6·37	0·609	5·74	0·397	8·02	0·48
30	45	7·00	0·460	8·99	0·745	8·16	0·570	10·33	0·66
40	60	7·66	0·461	10·82	0·857	10·37	0·740	13·30	0·99
50	75	8·18	0·463	12·49	0·916	12·01	0·850	16·69	1·20
60	90	8·60	0·463	14·06	0·930	13·62	0·991	18·45	1·36
70	105	8·54	—	14·81	—	14·14	1·00	21·60	1·60
80	120	—	—	15·29	—	14·70	1·01	21·81	1·66
90	135	—	—	15·65	—	15·48	1·04	24·50	1·73
100	150	—	—	16·35	—	16·28	1·11	26·08	1·84
110	165	—	—	16·45	—	17·85	1·14	20·83	1·87
120	180	—	—	—	—	17·92	—	27·55	1·93

* 15 minute intervals refer only to Exp. 4.
N = mg. acid-soluble N per 10 cc. solution.
P = mg. acid-soluble P per 10 cc. solution.

are shown in Table I A and curves showing the changes graphed as percentages of the total nitrogen and phosphorus are given in Figs. 1–4. The N/P ratios of the trichloroacetic acid precipitate at successive intervals of time were obtained by difference and are shown in Table II.

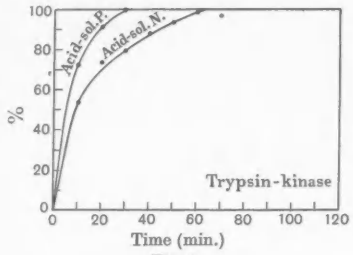


Fig. 1.

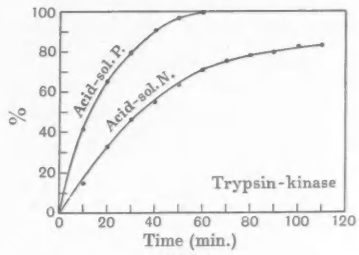


Fig. 2.

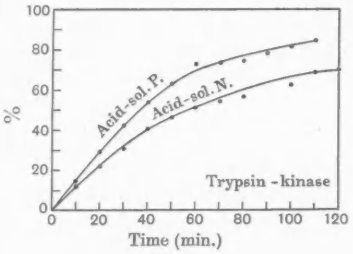


Fig. 3.

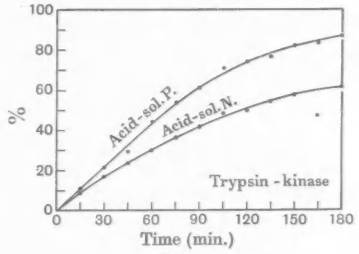


Fig. 4.

Hydrolysis by pepsin.

Caseinogen solution. This was made up in a manner similar to that for trypsin-kinase except that dilute HCl was used instead of alkali.

Enzyme solution. This was a 1 % filtered solution of granular pepsin (B.D.H.) the appropriate quantity of enzyme being obtained by dilution.

Table I B. *Pepsin (15 mg./10 cc. solution).*

Exp. No. / Fig. No.	5		6		7		8	
Total N (mg. in 10 cc. solution)	9·66		12·36		18·34		22·72	
Total P (mg. in 10 cc. solution)	0·515		0·652		0·959		1·23	
(Time mins.)	N	P	N	P	N	P	N	P
10	4·38	0·095	5·14	0·082	4·52	0·071	4·34	0·071
20	5·67	0·155	6·72	0·168	6·51	0·129	6·62	0·095
30	6·20	0·177	7·56	0·215	7·95	0·186	7·14	0·176
40	6·23	0·194	7·81	0·239	8·82	0·233	9·10	0·231
50	6·27	0·192	8·16	0·257	9·27	0·254	9·00	0·255
60	6·37	—	7·91	0·262	9·62	0·275	9·87	0·274
70	—	—	—	—	10·08	0·284	10·50	0·301
80	—	—	—	—	10·85	0·288	—	—
90	—	—	—	—	10·92	0·298	—	—
100	—	—	—	—	10·85	0·306	—	—
110	—	—	—	—	10·96	0·312	—	—

N = mg. acid-soluble N per 10 cc. solution.
P = mg. acid-soluble P per 10 cc. solution.

Buffer solution. The KCl-HCl mixture (p_H 1·8) of Clark and Lubs was used. The p_H optimum for pepsin as given by Northrop [1922] is 1·5–2·5 and our solutions throughout the course of the digestions were within this range.

Procedure: as for trypsin-kinase with the addition of one experiment in which six tubes containing the same amount of caseinogen but varying amounts of enzyme were incubated for 90 minutes. At the end of that time the amounts of acid-soluble nitrogen and phosphorus were estimated. This constituted Exp. 9.

Results. These are shown in Tables I B and II and in Figs. 5–8. The results of Exp. 9 are given in Table III.

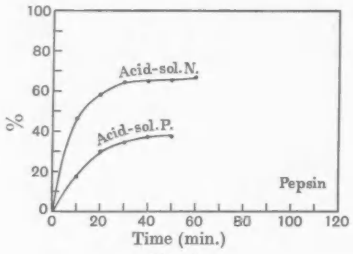


Fig. 5.

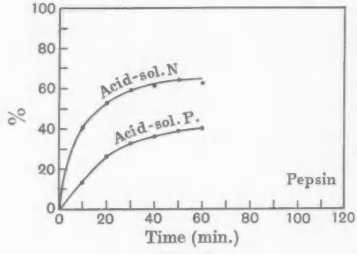


Fig. 6.

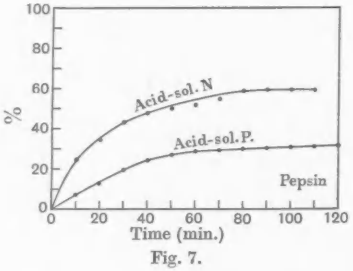


Fig. 7.

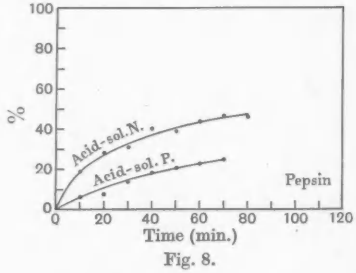


Fig. 8.

Table II. *N/P ratios of trichloroacetic acid precipitates.*

		Trypsin-kinase				Pepsin			
Exp. No.		1	2	3	4	5	6	7	8
Time (mins.)									
10	15*	32·6	29·9	19·5	20·2	12·6	13·1	15·6	15·8
20	30	58·2	40·3	21·1	20·8	11·1	12·2	14·3	14·1
30	45	∞	55·3	22·6	21·7	10·3	11·6	13·4	13·7
40	60	—	109·7	25·3	24·9	10·7	11·7	13·1	13·6
50	75	—	338·6	27·6	26·7	10·5	11·4	12·9	14·0
60	90	—	791·4	34·0	29·6	—	12·1	12·7	13·4
70	105	—	—	33·3	35·6	—	—	12·2	13·2
80	120	—	—	32·7	38·8	—	—	11·2	—
90	135	—	—	—	39·6	—	—	11·2	—
100	150	—	—	35·5	46·1	—	—	11·3	—
110	165	—	—	37·9	—	—	—	11·3	—
120	180	—	—	—	54·5	—	—	—	—

* 15 minute intervals refer only to Exp. No. 4.

Table III. *Exp. 9.*

Total N (10 cc. solution) 15·40 mg. Total P (10 cc. solution) 0·770 mg.
Each tube incubated for 90 minutes.

Pepsin (mg. per 10 cc. solution)	Acid-soluble N		Acid-soluble P		N/P ratio of trichloroacetic acid ppt.
	(mg. per 10 cc. solution)	Percentage total N	(mg. per 10 cc. solution)	Percentage total P	
5	6·70	43·5	0·107	14·0	13·12
10	7·96	51·7	0·184	24·0	12·70
15	8·85	57·5	0·248	32·0	12·54
20	8·94	58·1	0·276	36·0	13·08
25	9·47	61·5	0·306	40·0	12·77
30	9·60	62·3	0·315	41·0	12·75

Discussion.

The graphs show the relative rates of liberation of acid-soluble nitrogen and phosphorus during the digestion of caseinogen. In no case, either with trypsin-kinase or with pepsin, is it found that acid-soluble nitrogen and phosphorus are produced at the same rate, and it is therefore apparent that the first step in the degradation of caseinogen by proteolytic enzymes does not result in products which are completely soluble in trichloroacetic acid. This is quite contrary to the assumption made by Northrop [1924]. The fact that Northrop's assumption is unjustified is shown more clearly by Table II, in which are summarised the N/P ratios of trichloroacetic acid precipitates at successive intervals of time. It is quite apparent from these ratios that solubility in trichloroacetic acid is no index of the first stage of the degradation of caseinogen either by trypsin-kinase or pepsin. The caseinogen molecule can undergo very considerable disruption before becoming completely soluble in trichloroacetic acid and Northrop's work on the kinetics of trypsin action will have to be reconsidered from this new point of view.

Haldane [1930], commenting on Northrop's work, draws attention to his finding that the effect of substrate concentration is quite different according as the total action is measured by change in conductivity or production of amino-nitrogen, or by the actual destruction of caseinogen. Haldane suggests that this result may be due to the fact that Northrop's trypsin solutions contained carboxypolypeptidase and aminopolypeptidase (erepsin). These enzymes do not attack molecules of magnitude greater than polypeptides and would therefore play no part in the change from acid-insoluble to acid-soluble. A second alternative is suggested by Haldane, namely that trypsin has a greater affinity for the degradation products of caseinogen than for the protein itself. Such a hypothesis would be necessary to explain Northrop's finding that the amount of substance rendered soluble in trichloroacetic acid was almost directly proportional to the concentration of caseinogen between 1·5 and 5 % if the substance precipitated by trichloroacetic acid were wholly caseinogen. In our experiments this was certainly not the case and moreover we have found that the rate of liberation of acid-soluble nitrogen falls off

slightly with increase in caseinogen concentration. From the point of view of enzyme kinetics it is necessary that Northrop's work should be repeated using a method which will separate caseinogen completely from its cleavage products.

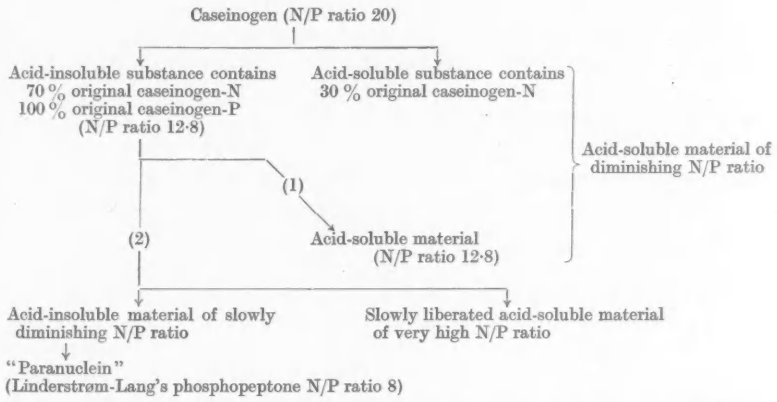
It will be seen from Figs. 1 to 8 that with trypsin-kinase acid-soluble nitrogen is liberated more slowly than acid-soluble phosphorus while, with pepsin, the reverse is true. This appears to indicate that the action of the two enzymes is fundamentally different, evidence for which can best be obtained by treating the data for each enzyme separately.

Trichloroacetic acid appears to separate peptic digests of caseinogen, in the initial stages of the hydrolysis of the protein, into two fractions: (1) insoluble in trichloroacetic acid and relatively rich in phosphorus (N/P ratio less than that of caseinogen), (2) soluble in trichloroacetic acid and relatively poor in phosphorus (N/P ratio greater than that of caseinogen). It is known that, at any one instant, several reactions are proceeding side by side during proteolysis and it might be expected that both the acid-soluble and acid-insoluble fractions would consist of mixtures of substances at various stages of degradation. That this is not the case, so far as the acid-insoluble material is concerned, may be ascertained from Table III. Here it is seen that, although the acid-soluble phosphorus has increased from 14 to 41 % of the total and the acid-soluble nitrogen from 44 to 62 %, the N/P ratio of the acid-insoluble material has remained practically constant. It is to be concluded therefore that, from the stage represented by tube No. 1 to that represented by tube No. 6 only one acid-insoluble substance was present and that the increase in acid-soluble material between these two stages resulted from the splitting of the acid-insoluble substance into two or more substances completely soluble in trichloroacetic acid.

Thus at least 86 % of the phosphorus of caseinogen can be split off in the form of a body insoluble in trichloroacetic acid and having N/P ratio of 12·8. From the fact that the precipitate from tube No. 1 had a N/P ratio of 13·1 we may infer that very little, if any, caseinogen still remained unattacked by the enzyme, and it is to be presumed that, in the 90 minutes which had elapsed since the beginning of the digestion, part of the non-caseinogen acid-insoluble material had acted as substrate and had contributed to the formation of acid-soluble phosphorus. It seems reasonable to conclude, therefore, that the initial action of pepsin on caseinogen is to split the protein into substances one of which is insoluble in trichloroacetic acid and contains all the phosphorus, while the other is soluble in trichloroacetic acid and contains no phosphorus. Although Exp. 9 demonstrates the cleavage of this phosphorus-containing acid-insoluble substance (N/P ratio 12·8) into fractions which are completely soluble in trichloroacetic acid it appears that this is not the only mode of degradation. This is shown by our data on the time-course of the N/P ratio of the trichloroacetic acid precipitate with varying concentrations of caseinogen as summarised in Table II. It will be seen that, in Exps. 5 and 6, the only acid-insoluble material present after 10 minutes' digestion is the

substance of N/P ratio 12·8 already mentioned. In Exps. 7 and 8 some substance of higher N/P ratio (probably unchanged caseinogen) is still present even after 10 minutes. Every experiment, however, shows a slow change in N/P ratio with time, a fact which indicates that here we have to deal with the cleavage of the acid-insoluble material (N/P ratio 12·8) into two fractions, (1) insoluble in trichloroacetic acid and low N/P ratio, (2) soluble in trichloroacetic acid and high N/P ratio. On the basis of viscosity measurements Linderstrøm-Lang [Holter *et al.*, 1932] has suggested that the mode of attack of the enzyme on its substrate may be different at different stages of digestion and our work would appear to confirm this. It may also be noted that a similar slow breakdown is shown in Table XII of Linderstrøm-Lang's paper. The phosphopeptone (paranuclein) investigated by Linderstrøm-Lang had a N/P ratio 8 and would appear to be a later stage of digestion than our acid-insoluble material of ratio 12·8. It was noted from Table II that the N/P ratio of the acid-insoluble material was tending towards the value obtained by Linderstrøm-Lang and it is possible that paranuclein is derived from our acid-insoluble substance by the second mode of breakdown suggested. It has to be remembered that Linderstrøm-Lang's material was characterised by being insoluble at acid reaction in the absence of trichloroacetic acid and that in his experiments the p_H of the system was not kept constant throughout the digestion. This may explain the fact that his phosphopeptone accounted for about 70 % of the total protein-phosphorus while in our Exp. 9 less than 60 % of the phosphorus is acid-insoluble and it is still in the form of the substance with a N/P ratio 12·8. The mode of breakdown seems therefore to depend on the composition of the system.

On the basis of these findings a provisional scheme for the hydrolysis of caseinogen by pepsin might be suggested as below:



(1) and (2) represent simultaneous modes of breakdown which may proceed at different relative rates according to conditions.

The course of the digestion of caseinogen with trypsin-kinase seems to be altogether different from that with pepsin. It will be seen from Figs. 1 to 4 that, with the former, the rate of liberation of acid-soluble phosphorus is greater than that of acid-soluble nitrogen. The action of the enzyme on caseinogen and on its immediate degradation products is characterised by the production of two fractions (1) insoluble in trichloroacetic acid and poor in phosphorus, (2) soluble in trichloroacetic acid and rich in phosphorus. As will be seen from Table II the N/P ratio of the trichloroacetic acid precipitate is increasing rapidly in the case of trypsin-kinase while it is decreasing slowly with pepsin. Since solubility in trichloroacetic acid may be taken as a rough guide to molecular size, it would appear that with trypsin-kinase phosphorus is liberated in comparatively small molecules but, with pepsin, in the form of large molecules. The differences in behaviour of the two enzymes can best be explained by the assumption that all the phosphorus of caseinogen is concentrated in one relatively small group. The phosphorus linkages would therefore appear to direct to some extent the action of proteolytic enzymes—trypsin-kinase effecting rupture of peptide linkages in proximity to phosphorus atoms and so confining the phosphorus to the cleavage product of lower molecular weight; the action of pepsin being relegated to peptide linkages distant from phosphorus atoms and giving rise to phosphorus-containing substances of high molecular weight. That groups within the molecule can exert a controlling influence over the action of enzymes is shown by the necessity for a free carboxyl or a free amino-group for the action of the polypeptidases [Grassmann and Dyckerhoff, 1928].

The further course of the hydrolysis with trypsin may be ascertained from Exps. 1 and 2 in which it was found possible to obtain all the phosphorus in an acid-soluble condition while about 20 % of the nitrogen remained insoluble. It is possible that the first action of trypsin-kinase on caseinogen results in the formation of two fractions, one acid-soluble and containing all the phosphorus, the other acid-insoluble and containing no phosphorus, the acid-insoluble phosphorus of the successive intervals of Exps. 1 to 4 being ascribed to unchanged caseinogen. The phosphorus-containing fraction would approximate to the size of Rimington's phosphopeptone which, it will be remembered, was isolated in amount representing over 50 % of the original caseinogen phosphorus. Rimington's compound is more likely to be a primary cleavage product of the caseinogen molecule than a secondary product resulting from the hydrolysis of a paranuclein as suggested by Linderstrøm-Lang [Holter *et al.*, 1932].

It is difficult to see how our results can be interpreted otherwise than as indicating that all the phosphorus is concentrated in one relatively small group in caseinogen, whether the latter is a single molecule or an association of molecules. If, as Linderstrøm-Lang suggests, caseinogen is an association of various proteins containing different amounts of phosphorus, it is impossible to account for the formation of one acid-insoluble substance containing over

86 % of the total phosphorus during peptic digestion. The possibility must therefore be seriously entertained that Linderstrøm-Lang's alcoholic-HCl has a chemical effect on caseinogen, and that his various fractions are not homogeneous.

Summary.

The action of trypsin-kinase and pepsin on the caseinogen molecule results in the formation of products which are not completely soluble in trichloroacetic acid.

With pepsin the rate of liberation of acid-soluble nitrogen is greater than that of acid-soluble phosphorus while with trypsin-kinase the opposite is true.

These results are explained on the basis of the existence of a caseinogen molecule with all the phosphorus concentrated in one comparatively small group.

The action of trypsin-kinase is considered to be on a peptide linkage in proximity to the phosphorus atoms thus confining all the phosphorus to the cleavage product of lower molecular weight. The action of pepsin is on a peptide linkage distant from the phosphorus atoms giving rise to a phosphorus-containing substance of high molecular weight.

The formation of Rimington's phosphopeptone is considered to be specific for the action of trypsin-kinase and that of paranuclein for the action of pepsin.

We have to thank Dr N. C. Wright for his interest in this work and for supplying us with caseinogen.

REFERENCES.

Clark (1928). The determination of hydrogen ions. (Baillière, Tindall and Cox, London.)
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Grassmann and Dyckerhoff (1928). *Z. physiol. Chem.* **175**, 18.
Haldane (1930). Enzymes. (Longmans, Green and Co., London.)
Holter, Linderstrøm-Lang and Funder (1932). *Z. physiol. Chem.* **260**, 85.
Linderstrøm-Lang (1928). *Z. physiol. Chem.* **176**, 76.
Northrop (1922). *J. Gen. Physiol.* **5**, 263.
—— (1924). *J. Gen. Physiol.* **6**, 417.
Rimington (1927, 1). *Biochem. J.* **21**, 204.
—— (1927, 2). *Biochem. J.* **21**, 1179, 1187.
—— and Kay (1926). *Biochem. J.* **20**, 777.
Van Slyke (1923). *Proc. World's Dairy Congress*, **2**, 1145.
Waldschmidt-Leitz (1923–24). *Z. physiol. Chem.* **132**, 181.
—— (1929). Enzyme actions and properties (trans.). (John Wiley and Co., New York.)
—— and Simons (1926). *Z. physiol. Chem.* **156**, 99.
Willstätter, Waldschmidt-Leitz, Dunaiturria and Künstner (1926). *Z. physiol. Chem.* **161**, 191.

CCXXXVII. NOTE ON THE OXIDATION OF TYROSINE, TYRAMINE AND PHENYLALANINE WITH HYDROGEN PEROXIDE.

By HENRY STANLEY RAPER.

From the Department of Physiology, The University, Manchester.

(*Received October 22nd, 1932.*)

Guggenheim [1913] has shown that the pods of the broad bean (*Vicia faba*) contain 3:4-dihydroxyphenylalanine, and Miller [1920] has isolated it from the Georgia speckled velvet bean (*Stizolobium deeringianum* Bort). It has also been found in insects [Schmalfuss and Müller, 1927; Przibram, 1922; Schmalfuss, 1927]. Recently, Schmalfuss and Heider [1931] isolated 3:4-dihydroxyphenylethylamine from the pods of the common broom (*Sarothamnus scoparius* Wimm) and found also that it was accompanied by tyramine.

The mode of origin in the plant or animal of these catechol derivatives is unknown, but it does not seem improbable that 3:4-dihydroxyphenylalanine is produced by the oxidation of tyrosine and 3:4-dihydroxyphenylethylamine by the oxidation of tyramine. It might be thought that tyrosinase would be the natural agent to bring about these oxidations, since it has been shown to produce *ortho*-dihydric from monohydric phenols [Pugh and Raper, 1927] and 3:4-dihydroxyphenylalanine has been isolated from the products of its action on tyrosine [Raper, 1926]. On the other hand, tyrosinase acts so rapidly on catechol derivatives that when produced by its action they would not tend to accumulate but be quickly oxidised further. This difficulty concerning their origin in living organisms could be overcome if tyrosinase consisted of two separate enzymes, one of which oxidised monohydric to *ortho*-dihydric phenols and the other oxidised only the *ortho*-dihydric phenols. If the first enzyme could act under conditions unsuitable for the second, then the catechol derivative under these conditions might accumulate. Pugh [1930] has shown, however, that there is no evidence that such a monophenol oxidase exists as a separate component of tyrosinase, and as this is the only enzyme known at present which oxidises monophenolic substances to catechol derivatives it is probable that some other agent is responsible for the oxidation of tyrosine and tyramine to their corresponding 3:4-dihydroxy-compounds.

In the course of experiments on the oxidation of *l*-tyrosine in dilute solution by hydrogen peroxide in the presence of various metallic catalysts it was observed that a trace of ferrous sulphate caused a fairly rapid oxidation of the amino-acid. Examination of the brown solution resulting from the

oxidation showed that it contained a substance giving a marked green colour with ferric chloride changing to reddish purple on the addition of sodium acetate. The substance responsible for this reaction was isolated and found to be *l*-3:4-dihydroxyphenylalanine, identical with the naturally occurring amino-acid. Under similar conditions it has also been found that tyramine is readily oxidised and yields 3:4-dihydroxyphenylethylamine, which was isolated as its tribenzoate.

Dakin and Herter [1907], studying the oxidation of phenylalanine, have shown that the use of hydrogen peroxide under more drastic conditions than were employed in the present experiments causes oxidation in both side-chain and nucleus. It seemed of interest to determine whether under milder conditions of oxidation the side-chain could be kept intact and the aromatic nucleus alone oxidised as with tyrosine and tyramine. To a small extent this has been found possible. By oxidising phenylalanine under the same mild conditions which were used with tyrosine and tyramine a small amount of tyrosine was obtained. This is of interest, since in alcaptonuria homogentisic acid has been shown to be produced from phenylalanine as well as from tyrosine, and Medes [1932] has recently described a condition in which phenylalanine is converted into *p*-hydroxyphenylpyruvic acid *via* tyrosine.

Several attempts have been made by the author to obtain homogentisic acid by the oxidation of *p*-hydroxyphenylpyruvic acid by hydrogen peroxide and iron, but they have all been unsuccessful.

These results show, therefore, that a possible mode of origin of *l*-3:4-dihydroxyphenylalanine and its corresponding base may be the oxidation of *l*-tyrosine and tyramine respectively by hydrogen peroxide under the catalytic influence of iron. They also demonstrate that tyrosine may be produced by the direct oxidation of phenylalanine and they add a few examples to the many which already exist of the probable importance of hydrogen peroxide as an oxidising agent in living organisms.

Experimental.

Oxidation of tyrosine.

To a solution of 2 g. tyrosine in 4 litres of water at room temperature were added 0·1 g. $FeSO_4, 7H_2O$ and 10 cc. (1·5 mols.) of 6 % H_2O_2. The solution soon became yellow and then gradually darkened to a brownish colour, depositing a little dark brown pigment. After standing for 3 days the solution was filtered and 10 g. lead acetate in 50 cc. water were added to the filtrate. This precipitates most of the brown pigment. The precipitate was filtered off and rejected. The filtrate on making definitely alkaline with ammonia gave a bulky precipitate. This was filtered off, washed a few times with cold water and then, after suspending in 200 cc. water, decomposed with H_2S. After removing the lead sulphide the solution was concentrated by distillation under reduced pressure until a good crystalline deposit had separated. The solution

was then heated to dissolve the deposit, transferred to a beaker, allowed to cool, neutralised to Congo red and concentrated to about 15 cc. in a vacuum desiccator over sulphuric acid. 0·32 g. of crystalline 3:4-dihydroxyphenylalanine was thus obtained; M.P. 275–80° (decomp.). By evaporating the mother-liquor to very small bulk a further crop of crystals of the amino-acid separated. In all 0·58 g. of crude dihydroxyphenylalanine was thus obtained. One recrystallisation from boiling water containing a trace of acetic and sulphurous acids gave a product with M.P. 279–80° (decomp.). This is the M.P. of the laevo-form of the amino-acid. It was greenish in colour due to traces of iron which are not completely removed in the process of separation *via* the lead salt. Attempts to remove the iron by decomposing the lead salt in presence of enough ammonia to render the solution alkaline resulted in a colourless product but with a lower M.P. 269–70°, this being due to racemisation. A sample of the racemised amino-acid obtained in this way, M.P. 268–9° (decomp.) was analysed: C, 55·2; H, 5·9; N, 7·1 %. $C_9H_{11}O_4N$ requires C, 54·8; H, 5·6; N, 7·1 %. By recrystallising the product of M.P. 279–80° three times from weakly acidulated water as above, crystals were obtained with only a slight colour. These were examined polarimetrically. 0·15 g. in 3·05 cc. *N* HCl gave $[\alpha]_{5461}^{18°} = -13·6°$. This is the value found by Harington and Randall [1931] for pure *l*-3:4-dihydroxyphenylalanine.

The amino-acid reduced ammoniacal silver nitrate in the cold and gave an intense green colour with ferric chloride.

Oxidation of phenylalanine.

3 g. inactive phenylalanine were dissolved in 3 litres of water and 0·15 g. $FeSO_4, 7H_2O$ added. 15 cc. of 6 % hydrogen peroxide were then introduced and the solution kept at room temperature until, after 4 days, the hydrogen peroxide had disappeared (chromic acid test). The solution had become deep brown and there was a brownish-black deposit. It was filtered, concentrated by distillation under reduced pressure to about 500 cc. and then 80 cc. of 20 % lead acetate were added together with 2*N* ammonia until no further precipitate came down. The lead precipitate was filtered off, washed and decomposed with H_2S in 600 cc. water. The PbS was removed by filtration, well washed and the filtrate and washings concentrated by distillation under reduced pressure to about 50 cc. and then, after neutralising to Congo red with ammonia, in a vacuum desiccator. A crystalline deposit was obtained which was recrystallised from boiling water, clarifying with charcoal. The substance crystallised in fine needles exactly like tyrosine; M.P. 287° (decomp.). Analysis, C, 59·3; H, 5·5; N, 7·6 %. $C_9H_{11}O_3N$ requires C, 59·7; H, 6·1; N, 7·7 %. The product was readily oxidised by tyrosinase from mealworms giving the typical colour changes of tyrosine. It also gave Millon's reaction. *o*- and *m*-Tyrosine both have a lower M.P. than that of the oxidation product and are not oxidised by tyrosinase from mealworms [Abderhalden, 1928]. From 4 g. of phenylalanine 167 mg. of the crude and only 43 mg. of the recrystallised tyrosine, M.P. 287°,

were obtained. The mother-liquor from which the first crop of tyrosine was obtained gave a further crop of crystals on concentration consisting largely of tyrosine as judged by the M.P. (270° after recrystallisation). The final mother-liquor gave a reddish-violet colour with ferric chloride suggesting the presence of *o*-tyrosine, but this was not confirmed in a second experiment. It is doubtful therefore whether *o*-tyrosine is produced along with the relatively more abundant *p*-tyrosine.

Oxidation of tyramine.

To 2 g. tyramine hydrochloride in 2 litres of water 0·05 g. $FeSO_4,7H_2O$ was added and then 60 cc. of 1 % H_2O_2 (1·5 mol.). The solution was allowed to stand overnight at room temperature, after which the H_2O_2 had disappeared. It was brownish-coloured and gave a marked catechol reaction with ferric chloride. 10 cc. of sulphurous acid were added and the solution was concentrated by vacuum distillation to about 100 cc. 50 cc. of 20 % lead acetate were added and then 2*N* ammonia until no further precipitate was formed. The lead precipitate was filtered off by suction on a pulp filter, washed with a little water and the paper pulp and precipitate suspended in 400 cc. water. The lead compound was now decomposed with H_2S, the lead sulphide and pulp removed by filtration and washed with hot water. The filtrate and washings were concentrated by vacuum distillation to about 30 cc. and then, after addition of 1·5 cc. concentrated HCl, in a vacuum desiccator. The dark brown crystalline residue was extracted with 20 cc. hot alcoholic HCl, filtered and the filtrate concentrated in a vacuum desiccator. A small amount of a sticky brown substance separated and was filtered off. The filtrate was now taken nearly to dryness in the desiccator when a brownish crystalline mass separated. This was filtered off, washed with a little absolute alcohol and dried *in vacuo*; 0·45 g. was obtained. It gave a strong catechol reaction with ferric chloride and was presumably the crude hydrochloride of 3:4-dihydroxyphenylethylamine. It was dissolved in a few cc. of water and benzoylated, using 5 g. benzoyl chloride and 30 cc. 10 % NaOH. The benzoylated base which separated was shaken out with benzene. On distilling off the benzene a viscous residue remained. This was redissolved in 5 cc. benzene and allowed to stand overnight in order to remove any tyramine dibenzoate, which is only slightly soluble in benzene. None was obtained. The solution was now allowed to evaporate slowly until crystals appeared. The crystallisation was aided by the cautious addition of light petroleum. The crystals thus obtained were filtered off, washed with a mixture of 3 parts benzene and 1 part light petroleum and dried *in vacuo*; M.P. 136–7°. On recrystallisation twice from 80 % aqueous alcohol and again from benzene and light petroleum a product was obtained with M.P. 140–1°. This is the M.P. of the tribenzoate of 3:4-dihydroxyphenylethylamine [Schmallfuss and Heider, 1931]. Micro-analysis, C, 74·6; H, 5·15; N, 3·2 %; $C_{29}H_{23}O_5N$ requires C, 74·8; H, 4·9; N, 3·0 %.

SUMMARY.

By the action of hydrogen peroxide with ferrous sulphate as a catalyst it is shown that phenylalanine yields tyrosine, *l*-tyrosine yields *l*-3:4-dihydroxyphenylalanine and tyramine yields 3:4-dihydroxyphenylethylamine.

A grant in aid of this investigation from the Government Grant Committee of the Royal Society is gratefully acknowledged.

REFERENCES.

Abderhalden (1928). *Fermentforsch.* **9**, 117.
Dakin and Herter (1907). *J. Biol. Chem.* **3**, 419.
Guggenheim (1913). *Z. physiol. Chem.* **88**, 276.
Harington and Randall (1931). *Biochem. J.* **25**, 1028.
Medes (1932). *Biochem. J.* **26**, 917.
Miller (1920). *J. Biol. Chem.* **44**, 481.
Przibram (1922). *Biochem. Z.* **127**, 286.
Pugh (1930). *Biochem. J.* **24**, 1442.
—— and Raper (1927). *Biochem. J.* **21**, 1370.
Raper (1926). *Biochem. J.* **20**, 735.
Schmalfuss (1927). *Biochem. Z.* **187**, 467.
—— and Heider (1931). *Biochem. Z.* **236**, 226.
—— and Müller (1927). *Biochem. Z.* **183**, 362.

CCXXXVIII. THE METABOLIC ACTIVITY OF THE CELLS OF THE TRIGEMINAL GANGLION.

By ERIC GORDON HOLMES.

From the Pharmacological Laboratory, Cambridge.

(*Received October 26th, 1932.*)

We have so far but little information about the relative metabolic powers of the different parts of the central nervous system. Kinnersley and Peters [1930] have observed that the concentration of lactic acid differs in different parts of the brain of the avitaminous pigeon, though not in the different parts of that of the normal bird. Gavrilescu and Peters [1931] have found that different parts of the pigeon's brain take up oxygen at different rates. Some information is available [Holmes, 1930] about the metabolic activity of the different parts of the neurone. The white fibres in the central nervous system, and those which form the peripheral nerves, appear to differ very materially in their oxidative powers from what we may, for the moment, call the "cell complex," that is, the cell body, the dendrites, and the junctional or synaptic tissue. It was in the hope of analysing a little further the activity of this "cell complex" that the work here described was undertaken. In the brain and spinal cord itself, the cell is so closely associated with dendrites and, presumably, with synapses, that no mechanical separation is possible. In the posterior root ganglia, however, cells and fibres are found, but the dendrites and axons, which are both to all appearances ordinary medullated fibres, pass out of the ganglia without making contact with other neurones. These ganglia, therefore, afford material for the examination of the activity of the cell bodies, uncomplicated by that of non-medullated dendrites or synaptic tissue. On the other hand, to make use of this tissue for the study of the metabolism of nerve cells, and to draw deductions from the results as to the behaviour of nerve cells in general, involves the assumption that the cells are similar in metabolic behaviour to others in other parts of the central nervous system; and this assumption is not necessarily justified, for the cells are larger than, and differ in form from, most of the cells in the central nervous system. However, it must be remembered that the latter present an enormous variety of shapes and sizes, ranging from the large pyramidal Betz cells of the motor cortex and the rounded Purkinje cells of the cerebellum to the small pyramidal cells which are present in such large numbers in the grey matter as a whole.

The experiments present other difficulties. The most convenient tissue to use was found to be the trigeminal ganglia of sheep and oxen. These bodies

contain, besides nerve cells, considerable amounts of white fibres, and of connective tissue. It is difficult to assess accurately the exact proportion of cellular substance present. A glance at a fixed and stained section, however, shows that the cells occur in large masses, and that they are closely packed, and it seems unlikely that their metabolic activity could be seriously masked by the presence of the relatively inert white fibres and connective tissue. In the light of what follows, it is noteworthy that a brief inspection of a section of cerebral cortex stained, for instance, with methylene blue shows that even here the cell density is not very great. The cells are much smaller than those of the posterior root ganglia and are dotted about amid a mass, not in this case of white fibres, but of non-medullated processes. If, therefore, the trigeminal ganglion cells may be taken as typical nerve cells, we have a fair chance of examining their biochemical behaviour.

The experimental procedure was as follows. Sheep's heads (usually three), were obtained fresh from the slaughter house. The skulls were sawn open, and the brains removed, the Vth cranial nerve being cut through as near as possible to the brain. The trigeminal ganglia, which lie on either side of the cavernous sinus, surrounded by tough dura mater and bone, were exposed and removed by the use of saw, bone forceps, and scissors. The three divisions of the trigeminal nerve were cut through close to the ganglion. The six ganglia were chopped with scissors, and sampled. Portions of the cranial nerves, of white matter and of cerebral cortex were usually taken for comparison. The oxygen uptakes were measured in the usual way, in the Barcroft apparatus. The tissue was suspended in Ringer's solution buffered with 1/5 volume of $M/2$ sodium dihydrogen phosphate adjusted to p_H 7·6. On every occasion, the Barcroft cups were filled with oxygen. Whenever possible, 0·5 g. of tissue was placed in each apparatus.

The curves obtained were, on the whole, far less satisfactory than is usual when dealing with other parts of the central nervous system. This was due, probably (1) to the very low rate of oxygen consumption; (2) to the difficulty of sampling. The connective tissue, which binds together the elements of the ganglion is extremely tough. It is, in consequence, difficult to chop the organ satisfactorily, and the tissue tends to collect in lumps in the Barcroft cups. In spite of these difficulties, it is at once plain that the oxygen consumption of ganglion tissue is of a very low order, much the same, in fact, as that of the peripheral nerve of the same animal. Samples of cortex taken at the same time as the ganglion, showed the usual large uptake, and there is no reason to suppose that the ganglion cells had suffered more manipulative damage than did those of the cortex. Fig. 1 shows the oxygen uptakes of trigeminal ganglion, postganglionic cranial nerve (V), and cortex—sheep's tissue being used in each case.

In only one experiment was a much larger oxygen uptake than this recorded, this being a single experiment in which ox tissue was used, when the uptake after 150 minutes amounted to 421 mm.^{3} This was quite an excep-

tional observation, and even so, the oxygen consumed amounted to only about a quarter of that consumed by sheep's cortex.

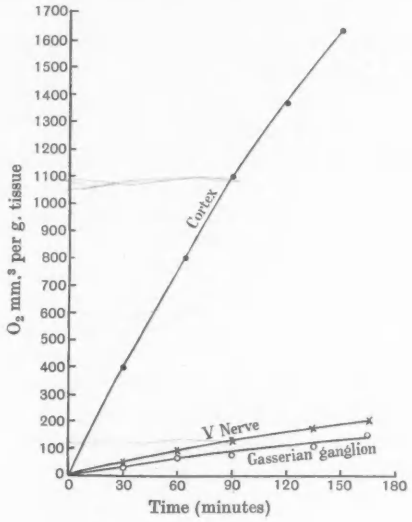


Fig. 1.

Table I.

Date	Tissue		Uptake after 150 mins. (mm.^{3}/g.)
1. iii. 32	Trigeminal ganglion (sheep)		132
8. iii. 32	,,		140
,,	,,		98
15. iii. 32	,,		170
,,	,,		128
22. iii. 32	,,		236
29. iii. 32	,,		178
		Average	154
8. iii. 32	Nerve (sheep)		86
15. iii. 32	,,		118
,,	,,		128
29. iii. 32	,,		114
		Average	112

Table I shows figures observed for total oxygen consumption both of ganglia and of cranial nerves. The values recorded for the uptake of the ganglia varied so much from experiment to experiment, even when the tissue was taken from the same mass of chopped ganglion, that it is impossible to say with certainty whether the oxygen consumption is or is not increased by the addition of lactate. That no large increase (such as may be observed with cortex) takes place is certain, but the experiments throw no light on the question as to whether or not a slight increase occurs. With succinate, on the

other hand, there is a very large increase. For instance, after 150 minutes, the oxygen uptake of sheep's trigeminal ganglion in Ringer and succinate ($M/20$) was 790 mm.^{3} per g. Quastel and Wheatley [1932] have shown that both grey and white matter readily change succinate to *l*-malate. The increase was therefore not necessarily due to the activity of the cells.

Since the oxidative powers of the nerve cells in question are evidently not of a high order, it was surprising to find that they readily gave a strong "Nadi" reaction. This is easily observed by freezing and cutting sections of the fresh ganglia, placing them in the "Nadi" reagent for a short time and then examining them under the low power of the microscope. The cells will be seen to be stained a deep blue colour, while the other tissue elements are nearly colourless. They, too, eventually become blue, but the colour is less intense than in the case of the cells and develops much more slowly. Incidentally, it was noticed that cooling to temperatures obtained with an ordinary acetone spray very much reduced the power of cortical tissue to consume oxygen.

The glycolytic power of the ganglion cells was also examined. A sample (0·7 g.) of tissue was placed in each of the two cups of a large Barcroft apparatus. One cup contained plain Ringer, the other Ringer containing 0·5 % glucose. The apparatus was filled with oxygen-free nitrogen and shaken for 3 hours in a thermostat at 37°. For purposes of comparison, samples of white matter and of cerebral cortex were treated in exactly the same way. The tissue, at the end of the experiment, was treated with trichloroacetic acid, made up to volume, filtered, and lactic acid estimated as usual. (Calcium hydroxide-copper sulphate precipitation: lactic acid estimated according to Friedemann, Cotonio and Shaffer [1927]).

The results are given in Table II. It will be seen that, in every case, less lactic acid is formed by the ganglia even than by white matter; cortex shows its usual (and marked) glycolytic power.

Table II. *Mg. of lactic acid formed per g. of tissue, after shaking for 3 hours anaerobically, with and without glucose.*

Ringer buffered with phosphate p_H 7·6.

Ganglion			White matter			Cortex		
Glucose absent	Glucose present	Formed from glucose	Glucose absent	Glucose present	Formed from glucose	Glucose absent	Glucose present	Formed from glucose
1·60	2·21	0·61	1·30	3·15	1·85	1·46	8·64	7·17
1·02	2·04	1·02	0·66	2·89	2·23	1·00	13·4	12·40
1·47	3·58	2·11	1·20	4·42	3·22	1·02	10·44	9·42
		1·24			2·43			9·66

These results show that mammalian sensory ganglia have feeble powers of oxidation and of glycolysis, when compared with the cerebral cortex. It has already been pointed out that the experiments on oxygen consumption are not without technical difficulties. Nevertheless, I venture tentatively to put

forward the hypothesis that, so far at least as oxidation and glycolysis are concerned, the chief seat of metabolic activity in the neurone is neither the medullated axon nor the cell body, but the dendritic and synaptic structures (supposing the latter to have a real and separate existence) which form such a large proportion of the bulk of the grey matter of the central nervous system.

It is of interest that Bethe [quoted by Evans, 1930], has found that the reflex mechanism of the tentacle of *Carcinus menas* continues to function, even after all the cell bodies have been cut away from the ganglion upon which the reflex depends, while Steinach [see Evans, 1930] has found that the posterior root ganglia of the rabbit continued to conduct impulses even after all the cells were dead and degenerated as a result of the cutting off of their blood supply. These findings, as Evans points out, suggest that the cell body is not essential for the conduction of impulses through the neurone, provided, of course, as in these cases, its destruction does not involve any actual interruption of continuity. But if the function of the neurone, as a unit, depends on its metabolic processes, these (or at any rate those of them which are important for function) must occur at some situation other than the cell body itself. We can eliminate, from previous studies, the medullated axon, and all that remains are the dendrites and synapses.

Summary.

The trigeminal ganglia of sheep show very poor powers of oxidation, although the cells contain indophenol oxidase. Their power of forming lactic acid from glucose is less than that of the white matter of the central nervous system. It is suggested that oxidation and glycolysis in brain occurs in the dendrites and synaptic structures, rather than in the cell bodies.

REFERENCES.

Evans (1930). Starling's Principles of human physiology. 242. 5th edition. (Churchill.)
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Gavrilescu and Peters (1931). *Biochem. J.* **25**, 1397.
Holmes (1930). *Biochem. J.* **24**, 914.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 711.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 725.

CCXXXIX. OBSERVATIONS ON THE VARIATION OF p_H OF BRAIN TISSUE.

By ERIC GORDON HOLMES.

From the Pharmacological Laboratory, Cambridge.

(*Received October 26th, 1932.*)

In previous communications [Holmes and Holmes, 1927; Holmes and Sherif, 1932], it has been demonstrated that the amount of lactic acid formed by the excised brain after incubation is dependent, within limits, on the level of the blood-sugar at the time of death, while the lactic acid found in brains which have been rapidly fixed in liquid air is constant in amount and independent of the blood-sugar, except when the latter has been reduced by insulin to a low level. It was thought that it would be interesting to see whether these changes in lactic acid content were associated with any demonstrable change in the p_H of the tissue.

Mice were used for the experiments; the blood-sugar was raised, when desired, by the intraperitoneal injection of glucose, or lowered by means of subcutaneous injections of insulin. The animals were decapitated by a single stroke of a pair of strong, sharp scissors, and the heads were either allowed to fall into liquid air (which prevents further lactic acid formation, even after thawing), or were incubated for 1 hour (sometimes 45 minutes) at 37°. For p_H determinations the brains were removed from the skulls, placed in a small beaker or specimen tube, and rubbed to a fine suspension with 5·0 cc. of Ringer's solution. The p_H of the suspension was determined by means of the Cole potentiometer, gold electrodes and quinhydrone being employed. The standard acetate buffer was checked against a calomel cell. While the values obtained probably do not accurately represent the actual p_H of the tissue during life, there seems no reason to doubt that the differences observed were significant; and the results showed a very considerable constancy. It is curious that, in spite of the fact that the determinations were not made in an atmosphere containing a proportion of CO_2, yet the values observed were consistently lower (*i.e.* the tissue was more acid) than it could reasonably be expected to have been during life. The results are arranged in six groups, as follows.

(1) Animals with normal blood-sugar, decapitated and the heads frozen.

(2) Animals with normal blood-sugar, decapitated and the heads incubated.

(3) Animals decapitated after glucose injection, and the heads frozen.

(4) Animals decapitated after glucose injection, and the heads incubated.

(5) Animals decapitated during insulin hypoglycaemia, and the heads frozen.

(6) Animals decapitated during insulin hypoglycaemia, and the heads incubated.

It will be seen that the values observed in groups (1) and (3) are about the same. There is a fall (increase of acidity) on incubation in all cases, but in group (4) it is definitely greater than in group (2); in group (6) it is slightly less than in group (2). The values in group (5), where the brains were frozen during hypoglycaemia, are definitely higher (less acid) than in (1) (normals). In a certain number of cases in groups (5) and (6), estimations of the blood-sugar were performed, the blood from several animals, killed at the same time, being pooled for this purpose. It will be seen from the tables that, in general, the lower the blood-sugar, the higher is the p_H, both in brains frozen immediately and in those which have been incubated.

All the estimations of p_H were carried out at room temperature, which varied from 10 to 18°, in different experiments. The correction which would have to be applied, according to Adair, Cordero and Shen [1929], who were working with blood, to correct the values for this would be in such a direction as to make the values for 37° even more acid than those actually observed. In a few experiments which were performed at different temperatures, this

Table I.

Group (1). Normal animals. Decapitated, and heads dropped directly into liquid air.

Date	p_H observed
5. v. 32	6·868
,,	7·035
,,	6·868
12. v. 32	6·823
,,	6·858
,,	6·888
,,	6·892
,,	6·920
18. v. 32	6·923
Mean	**6·897**

Group (2). Normal animals. Decapitated, and heads incubated at 37° for 2 hours (Exp. of 5. v. 32) and 45 mins. (Exps. of 6. v. 32 and 19. v. 32).

Date	p_H observed
5. v. 32	6·754
,,	6·763
6. v. 32	6·665
19. v. 32	6·670
	6·677
Mean	**6·704**

Group (3). Animals given 0·5 cc. of 40 % glucose in water by intraperitoneal injection. Decapitated after 15 mins.; heads dropped directly into liquid air.

Date	p_H observed
30. v. 32	6·984
,,	6·984
,,	6·958
Mean	**6·975**

Group (4). Animals given 0·5 cc. of 40 % glucose in water by intraperitoneal injection. Decapitated after 15 mins.; heads incubated at 37° for 45 mins. (Exp. of 6. v. 32) and 60 mins. (Exp. of 30. v. 32).

Date	p_H observed
6. v. 32	6·510
,,	6·440
,,	6·391
30. v. 32	6·408
,,	6·454
,,	6·464
Mean	**6·444**

Table I (*contd.*).

Group (5). Animals starved for 24 hours. Insulin (1 unit) injected subcutaneously. Decapitated after various intervals of time. Heads dropped directly into liquid air.

Date	Interval before killing	Symptoms	p_H observed	Blood-sugar mg./100 cc.	Mean p_H for given blood-sugar
13. v. 32	2 hours	+	6·978	—	
,,	,,	+	7·042	—	
,,	,,	+	7·037	—	7·029
,,	,,	+	7·037	—	
,,	,,	+	7·055	—	
18. v. 32	15 mins.	−	6·976		
,,	,,	−	7·037	61·2	6·955
,,	,,	−	6·853		
,,	45 mins.	−	6·966		
,,	,,	−	7·028	46·3	7·005
,,	,,	−	7·023		
,,	1 hr. 55 mins.	+	7·063		
,,	,,	+	7·073		
,,	,,	+	7·041	48·5	7·077
,,	,,	+	7·019		
,,	,,	+	7·079		
,,	,,	+	7·188		

Group (6). Mice starved for 24 hours. Given insulin as indicated in table. Decapitated, and heads incubated 1 hour at 37°.

Date	Insulin units	Interval before killing	Symptoms	p_H observed	Blood-sugar mg./100 cc.	Mean p_H for given blood-sugar
19. v. 32	0·1	15 mins.	−	6·855		
,,	,,	,,	−	6·982	74	6·931
,,	,,	,,	−	6·955		
,,	,,	45 mins.	−	6·977		
,,	,,	,,	−	6·931	39	6·954
,,	0·1 + 2·0	2·0 units given 3½ hrs. after 0·1 unit; killed after 4½ hrs.	+	6·923		
,,	,,	,,	+	6·923	26	6·934
,,	,,	,,	+	6·957		

was found to be the case for the tissue under investigation. No doubt, therefore, some change takes place rapidly in the tissue, immediately after excision, which renders it much more acid than it is during life. This does not necessarily invalidate deductions drawn from differences observed in the p_H of brains of animals which have been subjected to varying experimental treatment, and the results of the experiments do, in fact, fall into line with the previous observations on the behaviour of the brain-lactic acid. It is especially interesting that the values for frozen hypoglycaemic brains are actually higher than those for frozen normal ones, and this may possibly indicate that there is some actual p_H difference during life.

The actual decrease in p_H observed on incubation of the brains is shown in the following summary:

	Frozen	Incubated	Difference
Hypoglycaemic	7·029	6·934	0·095
Normal	6·897	6·704	0·193
Hyperglycaemic	6·975	6·444	0·531

It has previously been shown [Holmes and Sherif, 1932] that there is a rise in lactic acid on incubation of the brains, and that this rise is least with hypoglycaemic, greater with normal, and greatest with hyperglycaemic animals. It is clearly of interest to discover whether these changes in lactic acid content of the brain, which are known to occur, would, in fact, give rise to changes in p_H of the order here observed. Figures are available, both from the experiments already reported and from others performed since, for the increase in lactic acid to be expected in each of the three sets of conditions. In addition, the change in p_H to be expected from a given increase in lactic acid has been determined in the following way. The brains of 3 mice, weighing together approximately 1 g., were suspended in 10 cc. of unbuffered Ringer's solution, and the p_H was determined. 0·01 *N* lactic acid was added to the suspension in amounts of 0·05 cc. at a time, from a micro-burette, and the p_H determined after each addition. Titration curves were constructed, in this

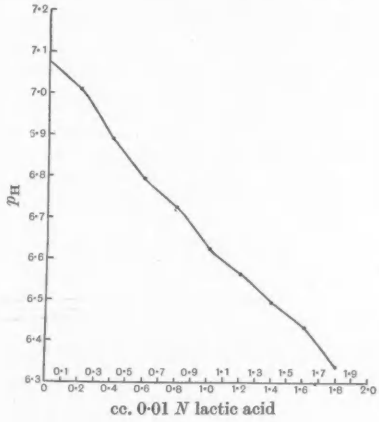


Fig. 1.

way, for the brains of normal and hypoglycaemic animals, both after fixing in liquid air and after incubation. As was to be expected, the initial p_H differed in the different cases, so that the curves in various experiments did not coincide. They were, however, very nearly parallel with each other. Fig. 1 is a curve constructed from the mean values of six of these titration curves. Table II shows the expected increase in lactic acid, the observed fall in p_H, and the fall in p_H read off from the titration curve.

Table II.

	Increase in lactic acid mg. per g. tissue	Increase as cc. 0·01 *N* acid	Fall of p_H (from curve)	Fall of p_H (observed)
Hypoglycaemic	0·16	0·177	0·093	0·095
Normal	0·54	0·60	0·257	0·193
Hyperglycaemic	1·39	1·54	0·640	0·531

For convenience, the increases in lactic acid are calculated in terms of cc. of 0·01 *N* acid per g. of tissue.

In the case of the hypoglycaemic animals, the agreement is good; the rise in lactic acid is small, but the relevant part of the titration curve (p_H 7·029 onwards) is steep. In the other two cases, all that can be said is that the changes observed and the changes calculated are of the same order.

Summary.

The p_H values, determined *in vitro*, of mouse brains which have been fixed in liquid air are higher in the case of hypoglycaemic animals than of normal or hyperglycaemic ones. In all cases the p_H of incubated brains is lower than that of those which are frozen immediately.

The changes in p_H observed are of the same order as those calculated from the titration curve of brain tissue and lactic acid, allowance being made for the amount of lactic acid which would be liberated in the various circumstances considered.

REFERENCES.

Adair, Cordero and Shen (1929). *J. Physiol.* **67**, 288.
Holmes and Holmes (1927). *Biochem. J.* **21**, 412.
—— and Sherif (1932). *Biochem. J.* **26**, 381.

CCXL. FURTHER OBSERVATIONS ON THE VOLATILE CHLORINE OF BLOOD.

By SAMUEL MORRIS and NOAH MORRIS.

From the Hannah Dairy Research Institute, Ayr, and the Biochemical Laboratory, Department of Paediatrics, Hospital for Sick Children, University of Glasgow.

(*Received October 20th, 1932.*)

In a previous paper [Morris and Morris, 1930] it was shown that drying of blood at 105° for a period of 4 hours led to a diminution in the chlorine content and it was suggested that the chlorine lost by drying was probably contained in a volatile organic compound. Sunderman and Williams [1931] published figures indicating that while a loss of chlorine occurred on drying blood and tissues it could be prevented by adding water to the dried blood and allowing the mixture to remain for 3 hours prior to the estimation of the chlorine. They further showed that the addition of olive oil to a solution of sodium chloride with subsequent drying led to the loss of a certain amount of chlorine which also could be prevented by the addition of water after drying and prior to the estimation, and concluded that in the case of blood the loss of chlorine was only an apparent one, being probably due to the effect of occlusion of the chlorine by fat. Quagliariello and Mazza [1931], on the other hand, state that the loss of chlorine on the heating of blood is due to drying and loss of HCl and is therefore no indication of the presence of organic chlorine compounds. Hogartz [1931] found no evidence of organic compounds of chlorine by extraction methods involving the use of alcohol and ether.

In our previous paper the results of experiments were given showing that the chlorine lost from the dried blood could be recovered almost quantitatively although not in the form of free chlorine or hydrochloric acid. In view of the results of Sunderman and Williams with olive oil, it was thought necessary to confirm our previous results and study the variations in the amounts of chlorine volatilised under different conditions.

Effect of drying blood at 105°.

Each sample of blood was divided into three equal parts, and the Cl content of the first was determined immediately by the Van Slyke method. The remaining two were dried for 4 hours at 105°; thereafter one was analysed by

the Van Slyke method and the other was completely ashed and its Cl content estimated by the same method. Table I indicates that practically the same diminution was obtained by drying whether the Van Slyke method was used directly or preliminary ashing was employed. It seems clear therefore that the decrease in the Cl value was not the result of occlusion.

Table I.

Blood no.	Chlorine milliequiv./litre. No preliminary drying	Preliminary drying	Preliminary drying and ashing
35	89·00	71·90	68·50
36	89·60	71·90	72·30
37	87·10	77·00	76·00

Effect of oxygen on the volatilisation of the chlorine from blood.

To study the effect of oxygen, samples of the same blood were dried in ordinary air, air deprived of oxygen and in an atmosphere of pure nitrogen. The air was deprived of oxygen by passing it through two flasks of pyrogallol prior to its entry into the drying flask. The atmosphere of nitrogen was supplied by heating NH_4NO_2. Table II shows that in the absence of oxygen no loss of chlorine is produced by drying at 105°. Furthermore, in the presence of air, addition to the blood of a strong oxidising agent such as potassium persulphate leads to an increased loss of chlorine on drying (Table III). This suggests that the presence of active oxygen facilitates the formation of the volatile chlorine compound.

Table II.

Blood no.	No drying	Preliminary drying			
		Ordinary air	Dry air	Dry air deprived of oxygen	Nitrogen
46	86·2	72·4	72·8	86·2	86·0
47	79·8	70·1	70·0	78·6	79·1
48	84·5	69·9	70·4	82·9	84·8
49	88·9	74·5	73·9	87·9	88·3
50	79·8	64·8	64·8	78·7	79·6
51	86·5	75·6	75·5	86·0	86·1

Table III. *Effect of addition of potassium persulphate to blood on the chlorine content after drying.*

Blood no.	No drying		Preliminary drying	
	Blood	Blood + $K_2S_2O_8$	Blood	Blood + $K_2S_2O_8$
51	85·66	85·60	73·00	62·30
52	85·20	85·25	69·95	59·24
53	87·68	87·72	65·36	57·71
54 (serum)	115·85	115·81	114·32	103·61

Effect of drying blood in a desiccator (Table IV).

Sunderman and Williams showed that drying blood over P_2O_5 also involved a loss of Cl and concluded that the essential factor was the loss of moisture. We studied the effect of drying blood for 48 hours over H_2SO_4: from some flasks oxygen was removed, others were filled with nitrogen, while others contained sodium cyanide solution. The effect of drying over H_2SO_4 was to produce a

Table IV. *Effect of drying blood in desiccator at room temperature (18°) in atmospheres of* (a) *air,* (b) *air deprived of oxygen,* (c) *nitrogen,* (d) *air, with NaCN added to the blood and* (e) *air, with $K_2S_2O_8$ added to the blood.*

Blood no.	No drying	Preliminary drying in oven at 105°	Air	Air deprived of oxygen	Nitrogen	Ordinary air; NaCN added to blood	Ordinary air; $K_2S_2O_8$ added to blood
(*a*) 46	86·2	72·4	78·8	86·2	86·0	85·5	71·1
(*b*) 47	79·8	70·1	74·9	79·5	79·8	79·8	71·9
(*c*) 48	84·5	69·9	74·9	84·0	84·1	84·0	67·3
(*d*) 49	88·9	74·5	80·6	89·0	87·9	87·8	76·0
(*e*) 50	79·8	64·8	71·4	78·8	78·9	78·4	60·6

diminution in the amount of chlorine recovered, although the loss was not so great as when the blood was dried at 105°. The addition of potassium persulphate increased the loss to at least that found on drying at 105°. The absence of oxygen from the flask or the presence of cyanide, which inhibits oxidation, prevented any loss of chlorine. It is clear therefore that the withdrawal of moisture in itself is not responsible for the diminution in chlorine content. It should here be stated that if the cyanide-blood mixtures are dried for over 48 hours the cyanide is converted into HCN and completely volatilised and there may occur some oxidative process with consequent loss of chlorine (Table V).

Table V. *Effect of drying blood with NaCN in desiccator for varying periods.*

		Preliminary drying		
Blood no.	No drying	Duration of drying in hours	Blood alone	Blood + NaCN
46	86·2	24	82·4	85·9
—	—	48	80·6	86·0
—	—	72	72·4	85·5
—	—	120	72·9	80·6
—	—	168	72·1	72·8

Table VI. *Effect of drying tissues* (a) *at 105°,* (b) *in desiccator and* (c) *with acetone and ether.*

Tissue (rabbit)	No drying	In oven at 105°	In desiccator	By acetone and ether
Heart	35·4	27·5	32·6	35·8
,,	33·8	26·2	30·1	35·0
Kidney	49·2	31·8	40·4	48·8
,,	54·1	36·5	46·2	54·0
Muscle	12·2	8·1	10·4	12·2
,,	16·4	11·9	14·0	16·0
,,	10·5	7·0	8·8	10·5

Effect of drying tissues under various conditions.

Prior to determination of their Cl content tissues were dried either in the oven at 105° or over concentrated H_2SO_4 or by Best and McHenry's acetone and ether method [1930]. The results (Table VI) fully corroborate the blood analyses, indicating that oxygen is essential for the volatilisation of the chlorine and that more chlorine is lost at a temperature of 105° than as a result of drying in a desiccator.

Effect of addition of olive oil to NaCl solution.

It seemed to us possible that the loss of chlorine on drying a mixture of olive oil and NaCl [Sunderman and Williams, 1931] might be due to reaction between the NaCl and unsaturated fatty acids.

This view was supported by the fact that heat is necessary to produce a loss of Cl from the olive oil-NaCl mixture. Further, the use of a fully saturated acid such as palmitic instead of olive oil should lead to no diminution of Cl content on drying the fatty acid-NaCl mixture at 105°. Experiments show that heating of palmitic acid-NaCl mixtures at 105° leads to no decrease in the Cl.

The only difference between blood and the olive oil-NaCl mixture as far as the loss of chlorine is concerned is the fact that the chlorine is lost from the latter mixture only after heating to over 100° whereas it can be driven off from the blood on drying in the desiccator without the aid of heat. This, however, is insufficient evidence that chlorine is present as an organic compound in blood. In order to demonstrate the presence of such a compound in blood it is necessary to extract it.

Organic solvents such as ether, benzene and toluene were found to be useless for the extraction of any chlorine. It was thought that a mixture of alcohol and ether might be efficient. Alcohol, however, mixed with the water present in blood and carried into solution some of the inorganic chlorine. It was therefore necessary to remove water from the blood by some method other than heat. For this purpose a modification of Best and McHenry's method for drying tissues was used. This consisted in the addition of acetone to blood, filtering, and washing four times with acetone in excess until no chlorine appeared in the acetone filtrate and then three or four times with ether. Air was then drawn over the residue by a suction pump, until a dry powder was obtained. The powder was removed to a flask and a mixture of equal parts of absolute alcohol and anhydrous ether was added. The powder was shaken with the alcohol-ether mixture for three periods of 15 minutes and then allowed to stand overnight. The alcohol-ether extract was then filtered off into a pyrex tube, and the alcohol and ether were evaporated off at a low temperature. Chlorine was invariably found to be present in the extract when this was treated with silver nitrate and nitric acid. So far we have been unable to recover more than 80 % of the amount lost on heating blood. With tissues the same method was employed after they were finely minced.

The alcohol-ether mixture does not extract chlorine from inorganic chlorides unless water is present. This we have repeatedly confirmed for the alcohol-ether mixtures we have been using.

It seemed possible, however, that the blood might have been incompletely de-hydrated by the acetone-ether treatment, and that the chlorine extracted was due simply to the inorganic chlorides being dissolved in the alcohol-ether mixture owing to the presence of moisture. To test this possibility a series of experiments was undertaken in which a synthetic mixture analogous to blood was used, containing 16 g. of protein (egg-albumin, serum-albumin or caseinogen), 0·5 g. of olive oil, 0·5 g. of NaCl and 0·1 g. of glucose per 100 cc. water. The mixture was well stirred and allowed to stand for about 4 hours prior to extraction, by the method used for the blood. In no case was chlorine found in the alcohol-ether extract.

Further, it was shown in a previous paper [Morris and Morris, 1930] that the volatile chlorine of blood diminishes on adding acids to the blood, the diminution being dependent on the strength of the acid. The addition of a strong acid should therefore diminish the amount of organic chlorine extracted from blood by means of alcohol and ether. From the same sample of blood two lots of 100 cc. were taken to one of which was added 10 cc. concentrated HCl. Both were allowed to stand for 4 hours and then extracted by acetone and finally by alcohol and ether. In the blood-HCl sample only minimum traces of chlorine (1–2 milliequiv.) could be detected in the alcohol-ether extract. This experiment which was repeated on four occasions affords further confirmation of the fact that inorganic chlorine is not extracted by the method described.

In view of these facts it seems justifiable to conclude that there exists in blood an organic chlorine compound.

Summary.

1. The presence of oxygen is essential for the loss of chlorine to take place.

2. The heating of an olive oil-NaCl mixture at a temperature over 100° leads to loss of chlorine.

3. Evidence is brought to show that there is close similarity between the two systems, blood and olive oil-NaCl, as far as the loss of Cl is concerned, except that in the latter mixture heat is required whereas with blood drying in a desiccator is sufficient to cause volatilisation of the Cl complex.

4. A method is described whereby organic chlorine can be extracted from blood. The method depends on removal of water by acetone and ether and subsequent extraction of the chlorine by an alcohol-ether mixture.

The authors regret that owing to an unfortunate oversight there was omitted from their previous paper an acknowledgment that the work therein described was commenced by one of them (S. M.) while in the Chemical Department of the Pennsylvania Hospital, Philadelphia.

We wish to express our thanks to Prof. G. G. Henderson, Prof. G. B. Fleming and Dr N. C. Wright for their helpful criticism and to the Medical Research Council for defraying the expenses of the investigation and for a personal grant to one of us (N. M.).

REFERENCES.

Best and McHenry (1930). *J. Physiol.* **70**, 349.
Hogartz (1931). *Z. physiol. Chem.* **200**, 119.
Morris and Morris (1930). *Biochem. J.* **24**, 1716.
Quagliariello and Mazza (1931). *Boll. Soc. Ital. Biol. Sper.* **6**, 703.
Sunderman and Williams (1931). *J. Biol. Chem.* **92**, 99.

CCXLI. FAT METABOLISM OF THE HERRING. I. A PRELIMINARY SURVEY.

By HAROLD JOHN CHANNON and MOHAMED KAMEL EL SABY.

From the Department of Biochemistry, University of Liverpool, and the Marine Biological Station, Port Erin, Isle of Man.

(*Received October 28th, 1932.*)

The gross chemical changes which occur in the tissues of the herring in different phases of sexual maturity have been studied by a number of workers. Milroy [1906; 1908] in a study of the muscles and gonads of Loch Fyne herrings observed a rapid rise in the percentage of fat (ethereal extract of tissue dried at 60–70°) in the muscles during the several months prior to the time when rapid growth of ovaries and testes began. This rise was followed by a steady fall and the lowest percentage was reached after the fish had spawned some 3 months later. The percentage of fat in the ovaries was variable. Similar observations were made on fish from the East Coast of Scotland [Milroy, 1907]. In these studies sexual maturity, length and weight of the fish were considered. Johnstone [1920] as a result of successive annual investigations [Johnstone, 1915; 1918; 1919] of the muscle-fat (carbon tetrachloride extract of tissue dried at 100° for 24 hours) was able to construct curves showing that the fat content of the muscles of Manx herring, which was less than 5 % in May, rose to a maximum in July or August (30–35 %), and then fell rapidly after the fish had spawned. The shape of each curve was characteristic of each season. Bruce [1924] deduced that, at any given stage of sexual maturity, the fat content of herring muscle was increased by age. He also quotes some results of Secker on the amount of fat in the livers and gonads of a number of samples of Clyde herring.

Thus, although much is known of the fat content of various tissues of the herring at different seasons, investigations have hitherto been mainly concerned with the herring as food. They have established irrefutably the progressive rises and falls in the fat content of the edible portion of the fish, but in no case have the investigations been comprehensive enough to allow of deductions being made as to the sequence of changes in fatty constituents occurring in the different tissues of a fish of given age throughout the period of gonad growth.

The work described in this paper was undertaken in an attempt to obtain more exact information regarding the rise and fall in the amount of the fatty constituents of the different tissues from the time when the gonads were

empty through the various stages of gonad development until the fish had spawned. By studying the amounts of fatty acids and cholesterol of all the tissues throughout the season, it was hoped to throw some light on the processes of translocation of these substances to the gonads.

Apart from the muscle and gonads, therefore, the livers have also been studied. Further, in the herring there is present at certain stages of the season a quantity of fat attached to the outside of the alimentary tract. This "mesenteric" fat has also been included in the investigations and, for ease of description in the Tables, this fat has been described as belonging to the intestine.

Collection of samples.

Samples of fish were collected once weekly over a period of 14 weeks from June 9 to September 11, 1931, from the marketable catches landed at Port Erin and Peel. From the average catch, something like 60 fish were taken as a sample. From the latter number, after investigation of length, sex and state of maturity, two further samples were taken to represent as far as possible the average of all these factors, each sample consisting of 6 male and 6 female fish. The region from which the fish were taken lies within an area bounded by a line drawn parallel to the west and south-west coasts of the Isle of Man between Peel and Port St Mary, and 8 to 10 miles from the coast, which constitutes the Port Erin fishing ground.

Observations of length, sex, state of maturity of roe or milt on Hjort's scale, and age were made on each fish of every sample. The number of vertebrae in the whole samples (about 1600 fish) were counted. These results are published separately [Saby, 1932]. Length was measured from the snout tip to the end of the longest caudal fin ray in the dorsal fluke of the tail.

The degree of maturity was classified on Hjort's scale. The classes actually encountered were: I, virgin, II, maturing virgins or recovering spents; III, gonads filling one-half the body cavity; IV, gonads filling more than two-thirds of the cavity; V, full; gonads filling the body cavity; VII, spent.

For the determination of age, several scales from the anterior region near the tip of the pectoral fin were removed and dried in air. Each collection was preserved in a numbered envelope, and later the scales were cleared and the winter rings counted under a binocular microscope according to the method of Lea [1910].

After determination of its weight, each fish was carefully dissected, and the liver, gonad and alimentary canal and a portion of the muscle were removed. The livers and gonads were readily obtained, but it was not found possible to remove the alimentary tract without some loss of the adherent fat. The alimentary tract was severed at the oesophagus and taken out entirely. The stomach was then opened and cleared, and gentle pressure was applied to the intestine in order to squeeze out as much of the remaining food residues as possible. The muscle sample was taken by cutting a fillet of the edible flesh extending from just behind the pectoral fin to the tail region. The selected tissues were then stored in stoppered bottles under excess of acetone until the collection was completed (14 weeks), when they were brought to Liverpool and worked up during the following 8 months. Each week there were thus obtained bottles containing respectively the livers, gonads, alimentary tracts and muscle fillets from 6 male fish, and the corresponding samples from 6 female fish. Table I gives the data concerning all the samples.

The determination of fatty acids and the unsaponifiable fraction.

Because of the labour and expense involved, it was decided in the first place to work up the samples from alternate weeks; the results obtained were so definite as to make it unnecessary to investigate the remainder.

The acetone was removed from each sample by filtration and the residue was repeatedly warmed with fresh samples of acetone, filtration and grinding of the residue in a mortar following each extraction. The combined acetone filtrates were evaporated to dryness *in vacuo*. The dry powdered residue was then extracted with ether (Soxhlet, 8 hours). The ethereal extract was added to the residue from the acetone, and the whole evaporated to dryness. The residue obtained

Table I.

Date	Sex	Length (cm.)									Age (years)							Maturity (Hjort's scale)						Wt. of the 6 fish	Wt. of livers	Wt. of gonads	Wt. of alimentary tract
		21	22	23	24	25	26	27	28	29	2	3	4	5	6	7	8	I	II	III	IV	V	VII	g.	g.	g.	g.
June 9	♂	.	1	2	2	1	.	.	.	.	3	3	.	.	.	.	.	3	3	.	.	.	.	598	11·0	2·5	23·0
	♀	.	1	2	3	.	.	.	.	.	4	2	.	.	.	.	.	2	3	1	.	.	.	574	11·0	6·5	24·0
„ 17	♂	.	1	1	2	1	1	.	.	.	1	4	1	.	.	.	.	1	3	2	.	.	.	710	13·0	10·5	36·0
	♀	1	1	1	1	1	1	.	.	.	2	3	1	.	.	.	.	1	2	3	.	.	.	659	16·4	17·1	31·1
July 7	♂	.	1	1	2	1	1	.	.	.	2	2	2	.	.	.	.	1	.	3	2	.	.	777	10·7	27·0	42·0
	♀	.	1	2	1	1	1	.	.	.	3	2	1	.	.	.	.	.	1	4	1	.	.	760	14·5	22·4	40·0
„ 24	♂	.	.	2	2	1	1	.	.	.	2	3	1	.	.	.	.	.	.	.	2	4	.	848	9·0	91·0	26·5
	♀	.	.	1	2	2	1	.	.	.	1	4	1	.	.	.	.	.	.	2	1	3	.	831	17·7	57·0	30·0
Aug. 5	♂	.	.	1	2	2	1	.	.	.	1	3	2	.	.	.	.	.	.	1	.	5	.	888	9·0	128·0	22·5
	♀	.	.	1	3	1	1	.	.	.	2	2	2	.	.	.	.	.	.	1	1	4	.	841	16·0	63·0	25·0
„ 20	♂	.	.	1	2	1	1	1	.	.	1	2	3	.	.	.	.	.	.	.	.	6	.	873	5·5	158·0	13·0
	♀	1	.	.	2	1	1	1	.	.	1	3	1	1	.	.	.	.	.	.	1	5	.	866	13·6	106·0	16·0
Sept. 5	♂	.	.	.	.	.	.	3	3	.	.	.	2	2	1	1	.	.	.	.	.	6	.	1162	5·5	256·0	12·0
	♀	.	.	.	.	.	1	4	1	.	.	1	1	3	.	.	1	.	.	.	.	6	.	1102	16·0	212·0	15·0
„ 11	♂	.	.	.	.	.	2	.	3	1	.	.	1	4	1	.	.	.	.	.	.	.	6	915	12·0	Spent	11·0
	♀	.	.	.	.	.	1	3	.	1	.	.	1	3	1	.	1?	.	.	.	.	.	6	850	11·0	„	14·0

was then saponified by boiling with excess of alcoholic potassium hydroxide and, after removal of much of the alcohol, followed by dilution with water, the unsaponifiable fraction was obtained by 6 extractions with ether. The ethereal extract, after washing with water to neutrality, yielded, on evaporation and drying *in vacuo* at 100°, the unsaponifiable fraction. The combined soap solutions and aqueous washings were acidified with hydrochloric acid, and similarly extracted with ether. After washing, the ethereal extract was evaporated and the residue dried at 100° *in vacuo*. It was then dissolved in light petroleum and allowed to stand overnight. After filtration, the light petroleum solution yielded, on evaporation, the fatty acids, which were dried at 100° *in vacuo* and weighed. Iodine values [Rosenmund and Kuhnhenn, 1923] were determined on the fatty acids and unsaponifiable fraction, and the amount of cholesterol in the latter by the digitonin method.

Results.

The percentage yield of fatty acids.

The yields of fatty acids (g. per 100 g. of fresh tissue) are recorded in Table II.

Table II. *Percentage yield of fatty acids.*

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
June 17	11·43	10·76	30·85	22·32	10·00	10·66	5·14	2·61
July 7	14·48	15·47	40·34	38·30	13·71	17·19	5·64	4·79
„ 24	9·31	8·83	31·93	28·99	17·83	17·09	1·82	3·84
Aug. 5	13·12	10·92	23·59	21·10	17·54	14·58	1·58	4·35
„ 20	5·13	5·77	10·63	15·31	15·09	16·04	1·90	3·16
Sept. 5	2·59	3·92	1·47	2·85	12·73	14·24	2·12	4·15
„ 11	0·99	1·29	0·87	1·26	10·86	Lost	Spent	Spent

These figures show that in the 21 days between June 17 and July 7 there has been a very large increase in the percentage of fatty acids in the liver, intestine and muscle. The percentage increases over the values of June 17 are as follows: liver ♂ 27·0, ♀ 43·8; intestine ♂ 30·8, ♀ 71·6; muscle ♂ 37·1, ♀ 61·3. In the liver and intestine, both for male and female fish, July 7 marks the highest values obtained throughout the experiment. From this date onwards there is a steady decrease throughout the remainder of the period. Thus, in the last samples, the spent fish of September 11, the percentage of fatty acid in the livers has sunk to 0·99 and 1·29 for the males and females respectively, as against 14·48 and 15·47 % on July 7, showing that just over 90 % of the initial liver-fatty acids have been utilised or translocated elsewhere. The results with the intestinal fat are even more striking, for the fatty acids disappearing account for 97·9 and 96·7 % of those present on that date. The maximum percentage of fatty acids in the muscle appears to occur rather later, July 24, and the final percentage in the last sample in the male fish shows a fall of 39 % from the highest value. The final female sample was unfortunately lost; but the value for the previous collection of September 5 shows a fall of 17·2 %. The percentage fatty acid content of the gonads in the different samples shows no regular change, which is perhaps a somewhat unexpected result, although similar to that of Milroy [1908].

The results are expressed graphically in Fig. 1 where the marked initial rises in the percentage fatty acids of liver, intestine and muscle are clearly

seen, as are the gradual progressive losses of fatty acids from these tissues with gonad development. The approximately 70 % increase in the percentage of muscle-fatty acids in the first 38 days is noteworthy. (It is to be observed that in Fig. 1 the curves are all drawn on the same scale save those for the intestinal fat which, reaching a percentage of 40 and falling finally to about 1 %, necessitates a scale one-fifth as great.)

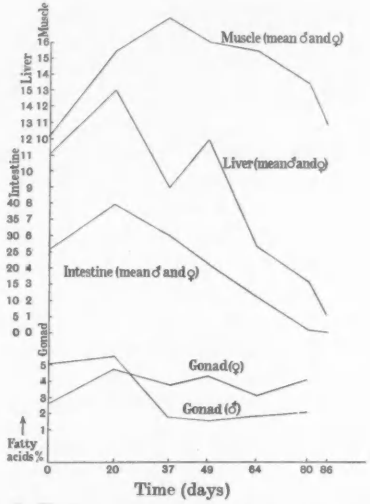


Fig. 1. Variation in the percentage of fatty acids in the tissues of the herring during the maturation period.

The percentage yield of unsaponifiable matter.

The yields of unsaponifiable matter (g. per 100 g. of fresh tissue) are recorded in Table III.

Table III.

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
June 17	1·09	0·81	0·54	0·61	0·19	0·19	0·43	0·47
July 7	1·04	0·94	0·58	0·61	0·15	0·15	0·63	0·52
,, 24	1·11	0·72	0·76	0·65	0·15	0·22	0·38	0·58
Aug. 5	1·13	0·67	0·60	0·45	0·17	0·18	0·45	0·54
,, 20	0·72	0·49	0·71	0·67	0·19	0·22	0·60	0·47
Sept. 5	1·40	0·41	0·92	0·66	0·13	0·16	0·66	0·51
,, 11	0·31	0·46	0·37	0·48	0·15	—	Spent	Spent

In Table IV the unsaponifiable matter is expressed as a percentage of the fatty acids. The unsaponifiable fractions consist largely of cholesterol, the average figures being liver 42 %, intestine 57 %, muscle 37 %, gonad 84·5 %.

Table IV.

Date	Liver ♂	Liver ♀	Intestine ♂	Intestine ♀	Muscle ♂	Muscle ♀	Gonad ♂	Gonad ♀
June 17	9·6	7·5	1·75	2·74	1·9	1·78	8·3	18·0
July 7	7·2	6·1	1·44	1·59	1·09	0·87	11·2	10·9
„ 24	11·9	8·2	2·38	2·24	0·84	1·29	20·9	15·1
Aug. 5	8·6	6·1	2·54	2·13	0·97	1·23	28·5	12·4
„ 20	14·0	8·7	6·68	4·37	1·26	1·37	31·6	14·9
Sept. 5	54·0	10·5	62·60	23·12	1·02	1·14	31·1	12·3
„ 11	31·3	35·7	42·53	38·09	1·38	—	Spent	Spent

From the figures recorded in Table IV it is clear that the rate of utilisation of the unsaponifiable fractions is very much less rapid than that of the fatty acids of the liver and intestine, which Table II shows are almost completely lost from those tissues by the time that maturation is complete. Thus, whereas in the liver on June 17 the percentage of unsaponifiable matter is about 1/12 that of the fatty acids, on September 11 it constitutes about 1/3, a fourfold increase. Similarly for the intestine, the ratio of unsaponifiable matter to fatty acids rises from its initial value to a final one something like 20 times as great. The high ratio in the gonads is to be expected, while the lack of regularity in these figures is consequent upon the variable percentages of fatty acids in the different samples, as recorded in Table II.

The iodine values of the fatty acids.

It is not necessary to reproduce the iodine values in full, because, save in the case of the gonads, the variations obtained were irregular and no deductions could be made from them. The extreme and mean values are recorded in Table V.

Table V. *Variations in iodine values of the fatty acids.*

	Highest ♂	Highest ♀	Lowest ♂	Lowest ♀	Mean of all ♂	Mean of all ♀
Liver	133	141	120	115	127	130
Intestine	124	122	91	91	107	109
Muscle	136	126	102	105	116	119

In the case of the gonads, however, the fatty acids showed a progressive increase in unsaturation with the development of maturity, and these figures are presented in Table VI.

Table VI. *Iodine values of fatty acids of gonads.*

	June 17	July 7	July 24	Aug. 5	Aug. 20	Sept. 5	Sept. 11
♂	128	151	162	158	195	209	Spent
♀	146	143	163	170	174	195	„

The high degree of unsaturation of the fatty acids just before spawning is noteworthy, as is also the fact that these fatty acids are very much more unsaturated than even those of the liver itself.

Superficially, the fact that the gonad-fatty acids have iodine values some 50 % greater than the fatty acids of any of the other tissues suggests that desaturation of the fatty acids is occurring, especially as for the last month at least the fish is not feeding. Alternatively, the high degree of unsaturation of the gonad-fatty acids might be due to preferential translocation of the more unsaturated acids from the liver. The first point to be mentioned, as will be shown later, is that at the time of maturation the gonads contain about 1 g. of total fatty acid, whereas the liver has a maximum fatty acid content of only about 0·3 g. which falls to about 20 mg. when maturation is complete. Since the iodine value of the liver-acids does not change significantly during the maturation period, and further, since the total loss of fatty acid from the liver is no more than 0·28 g., it is clear that the fatty acids which the liver has lost cannot in any way explain the presence in the gonad of 1 g. of fatty acid of iodine value 200. On the other hand, preferential transference of the more unsaturated acids from the muscle to the gonad could account for the high iodine value of the gonad-fatty acids, for the muscle of the average fish contains about 17 g. of fatty acids during the earlier period, of which about 4 g. are lost by the time that maturation is complete. These 4 g. of iodine value 116 would provide 464 available iodine units, of which the 1 g. of fatty acids in the gonads at maturity require only 200. Hence the loss of fatty acids from the muscle could account for the appearance of 1 g. of fatty acids of iodine value 200 in the ripe gonad, without the iodine value of the remaining muscle-fatty acids changing. Similar reasoning can be applied to the fatty acids of the intestinal fat. During the later stages of the maturation period the fish is calling on its reserves of fat for energy purposes. If the more unsaturated acids disappearing were translocated to the gonads and only the more saturated acids were being burnt, then results recorded in Table VI would be explained without invoking the aid of a desaturation hypothesis. On the other hand, it is possible that the gonad-fatty acids may represent the results of a desaturation process occurring in the liver or elsewhere, and evidence on this question is now being sought. Further, the possibility of a synthesis *in situ* must not be overlooked. The occurrence of these acids of high iodine value in the ovaries and testes may suggest that they are present as phosphatides, which the findings of Milroy [1906; 1908] and Bruce [1924] of an increasing phosphorus content in these tissues with maturation may imply.

Yield of sterol.

Cholesterol was not estimated in the unsaponifiable fractions until the samples of July 24. These are set out in Table VII. These figures show a regular decrease in the percentage of liver-cholesterol, while that of the muscle remains constant. The figures for the cholesterol content of the intestine are somewhat irregular.

On the other hand the contrasting results for the testes and ovaries are

worthy of mention. Whereas in the testes the amount of cholesterol is steadily increasing to a maximum prior to the spawning of the fish that of the ovaries shows but small and irregular changes with the approach of maturity.

Table VII. *Yield of sterol per 100 g. of fresh tissue (mg.).*

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
July 24	—	431	294	338	67	75	335	458
Aug. 5	559	366	316	351	68	65	369	438
„ 20	375	261	—	318	64	69	533	397
Sept. 5	263	187	492	297	—	66	615	410
„ 11	168	197	244	—	66	—	Spent	Spent

The absolute gains and losses of fatty acids and cholesterol by the tissues.

With tissues which are increasing or decreasing so rapidly in weight (gonad, liver, intestine), the expression of the fatty constituents as percentage figures gives no idea of the changes in weight which these constituents undergo. It is of considerable interest therefore to attempt to convert these percentage figures into weights, in order to gain some idea of the magnitude of the loss or gain of the fatty constituents by the various tissues in the successive stages of maturation. This raises the question as to whether it is safe to assume for the purposes of discussion of the results that fish drawn from one fishing ground over a limited time, if comparable as regards age and sexual maturity, may be regarded within rough limits as comparable in other respects, provided the inherent error of small random sampling be adequately recognised at the outset. It is of course definitely known that herring spawn at definite and often widely differing times in different latitudes. Thus herring in the Port Erin area always spawn in August and September, while in Western Scottish waters the period is January and February [Milroy, 1908], and other examples may be cited. Unless therefore the herring caught in a given area consist of a number of different races, in which the weight to age ratio varies considerably, it appears to us that it may not be unprofitable to compare the weights of the fatty constituents present in successive samples and to consider the changes in these weights as roughly representing the results of the metabolism during the intervening periods, provided the ages of the fish and the degree of sexual maturity which are known are comparable. (The results of counting the vertebrae of the 1600 fish from which our samples were drawn provide no evidence for the existence of different races [Saby, 1932].)

Fourteen samples in all were analysed over the period June 17 to September 11, each sample consisting of 6 fish, and we may now consider the ages of the fish constituting the different samples. It is seen (Table I) that in every one of the 10 samples from June 17 to August 20 there are present one 2-year old, two 3-year old and one 4-year old fish, *i.e.* in each sample 4 of the 6 fish are of the same age. Of the 60 fish, therefore, 40 are equally distributed as regards age in the 10 samples. Further, save for one

individual fish, the remaining 20 are also 2, 3 or 4 years old. We may take it also that any 3-year old fish in these 20 should play no part in rendering the weight relations incomparable. Of these there are 8. Hence it seems to us that of the 60 fish in the 10 samples only 12 should cause any possible serious variation in weight relationships. Further, in two samples there are two 2-year old fish, but these are offset by the presence of two 4-year old fish, and these samples also should therefore be comparable with the rest. Thus, of the 60 fish involved in these 10 samples, only 8 should cause any appreciable variation and, remembering that the difference in size of 2, 3 and 4-year old herring is not large, it seems to us that these 10 samples may be compared as a rough approximation. Further, the average length of the 6 fish in each of these 10 samples is 24, 23·5; 24·0, 23·8; 24·2, 24·5; 24·5, 24·3; 24·8 and 24·7 cm. over 65 days. This appears to confirm the reasoning regarding the even distribution as regards ages, and strengthens the ground for regarding the weight of fatty acids in the tissues of the fish of various successive samples as representing actual metabolic changes occurring between successive samplings. These samples are also in order of increasing maturity (Table I).

On the other hand, the last four samples of September 5 and 11 are not comparable with these 10, because, of the 24 fish involved, 18 are over 4 years old. The average ages of these samples are 5·3, 5·0, 6·0 and 5·2 years, and their average lengths 27·5, 27·5, 27·5 and 27·3 cm., whereas the extremes in the average length of the fish in each of the first 14 samples are 23·5 and 24·8 cm.

Although the weights on September 5 and 11 are thus vitiated it is of some importance to derive figures for these two dates, especially for September 5, which was the last day on which the fish were taken before spawning, and we think that a close approximation can be obtained.

Before proceeding to discuss this matter further it is to be pointed out that only fillets of the muscle were taken and hence the total muscles of each fish were not weighed at the time of collection, but the approximate weights of the muscles can be derived from Table I. In order to obtain a figure for the muscles of the herring, 8 fish of markedly varying weights and gonad development were dissected and the alimentary tracts, livers and gonads removed. The residue, consisting of muscle, head, tail and skeleton was weighed, W. The muscle was then torn away and the head cut off. The combined weight of the head and tail and the skeleton after washing in water and roughly drying represented 17·6 to 21·3 % of W. For the purposes of this discussion, therefore, the total weight of muscle may be taken as 80 % of the weight of the total fish less that of the alimentary tract, liver and gonad. Using this figure of 80 %, therefore, the figures on p. 2030 are thus derived from Table I.

The big increases in the muscle weight of the samples of September 5 and 11 are at once apparent.

We incline to the view that the percentage composition of fish in the same stage of maturity does not vary significantly with age. It is true that Bruce

	June 17		July 7		July 24		Aug. 5	
	♂	♀	♂	♀	♂	♀	♂	♀
Wt. of muscle	86·8	84·0	93·0	91·1	96·2	97·2	97·1	98·1

	Aug. 20		Sept. 5		Sept. 11	
	♂	♀	♂	♀	♂	♀
Wt. of muscle	92·9	97·3	118·4	114·5	119·0	110·2

[1924] deduces from his muscle analyses that the fat content of the muscle at any given gonad stage depends to some extent on age, but from the present work it is clear that any slight difference due to age is insignificant relatively to the influence of different states of sexual maturity. Now the muscles of the 6 samples preceding September 5 approximate in a sufficiently constant manner to 97 g. to make it accurate for our purposes for this weight to be taken for the samples of September 5 and 11. In Table VIII and Fig. 2 which follow, therefore, the total muscle-fatty constituents for these two dates has been derived by multiplying the found percentages not by the known weights but by the deduced figure 97.

No similar correction need be applied to the liver and intestine, for at this stage their respective weights are only about 2 g. and they contain only about 1 % of fat.

The last gonad weights, however, of September 5 are naturally in need of correction. Since this tissue is increasing in weight rapidly at this time, it is an uncertain proceeding to attempt to correct it, and therefore in the Tables which follow the figures for the gonads of September 5 appear in brackets, and the last portion of the curve is shown as a broken line.

It is of interest to note in passing that the weights of fatty acids in the gonads plotted against time lie on a straight line up to the date preceding September 5. If this straight line were continued to give a corrected figure for the value under discussion we obtain 0·66 g. as against the 1·18 g. given by the enhanced finding on the actual weight of the older fish. That this figure (0·66 g.) must be significantly low is shown by the cholesterol figures in Table IX. Reduction of the sterol figure of September 5 proportionately results in a sterol figure for the September 5 samples actually below that of August 20. This means that, if cholesterol and fatty acids are increasing at the same rate, the fatty acid figure must be considerably greater than 0·66 g.

There are now presented tables showing the variation in the weights of the fatty acids in the different tissues of the average fish.

The figures in Table VIII may therefore be taken as an approximate indication of the changes which occur in a 3-year old herring during the period of active growth of the gonads from an initial weight of 0·8 g. to a final weight of 39 g. when spawning occurs. The liver-fatty acids increase during the first 3 weeks from an initial value of 0·27 g. to the slightly higher figure of 0·31 g. For the remainder of the period, the liver steadily loses its fatty acids until in the week after spawning not more than 20 mg. remain. The intestinal fat

Table VIII. *Total weight of fatty acids in the different tissues of the average fish (g.).*

Date	Liver		Intestine		Muscle		Gonad	
	♂	♀	♂	♀	♂	♀	♂	♀
June 17	0·26	0·29	1·85	1·16	8·68	8·95	0·09	0·07
July 7	0·26	0·37	2·82	2·57	12·75	15·66	0·26	0·18
,, 24	0·14	0·26	1·40	1·45	17·16	16·61	0·27	0·36
Aug. 5	0·20	0·29	0·87	0·89	17·03	14·31	0·33	0·46
,, 20	0·05	0·13	0·23	0·41	14·02	15·62	0·50	0·56
Sept. 5	0·02	0·10	0·03	0·08	12·35*	13·82*	(0·90)	(1·47)
,, 11	0·02	0·02	0·02	0·03	10·54*	Lost	—	—

* Corrected as described on p. 2030.

increases similarly in the first 21 days to 2·7 g. and the whole of this amount is then steadily lost by the time that spawning occurs. The fatty acids of the muscle, 8·8 g., however, regularly increase for a rather longer time (about 5 weeks) and thus reach their maximum towards the half-way stage of the maturation process at 16·8 g. They then undergo a roughly similar rate of decrease to a weight of about 10·5 g. in the 13th week. Meanwhile the gonad-fatty acids commencing at 80 mg. increase steadily to a maximum prior to spawning. These rises and falls are illustrated in Fig. 2.

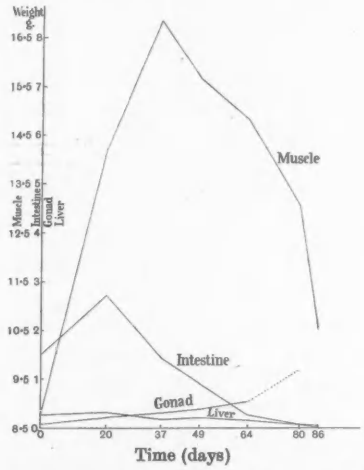


Fig. 2. Variation in weight of fatty acids in the tissues of the average fish during the maturation period (mean ♂ and ♀).

It is not possible to deduce the source of the gonad-fatty acids. It is perhaps of importance to observe that the fatty acids of the liver and mesentery have begun to decrease after 21 days although the muscle-fatty acids are rapidly increasing in the male fish and slightly in the female. This is the more striking when it is remembered that the increase in the muscle-fatty acids

during the next 17 days in the male is 4·4 g., and yet during this time the intestine has lost 1·4 g., and even the very small initial amount of fatty acid in the liver, 0·27 g., is tending to fall. It appears as though the intestinal fatty acids were serving as reserve fat for increasing the muscle-fat as required, but it is not easy to see why fat deposited as reserve fat should be mobilised in order to increase that of the muscles, unless the muscles were possibly playing some part in the preparation of those fatty acids for use possibly by the gonads. It is to be noted that no food was found in the fishes' alimentary tracts after the beginning of August, *i.e.* when the intestinal fat has begun rapidly to decrease. Equally curious also is the fact that the liver containing only 0·27 g. of fatty acid should lose the greater part of this small amount when such relatively vast quantities are available in the muscle.

The losses and gains of the tissues from the time of maximum fatty acid content to September 11 are as follows:

	Fatty acid (g.)	
	Loss	Gain
Liver	0·25	—
Intestine	2·65	—
Muscle	3·76	—
Gonad	—	0·98
Total	6·66	0·98

The net loss of 5·68 g. must have gone towards the supply of energy during the final period of abstention from food.

Table IX. *Total weight of cholesterol in the different tissues of the average fish (mg.).*

	Liver		Intestine		Muscle		Gonad	
Date	♂	♀	♂	♀	♂	♀	♂	♀
July 24	—	12·7	12·9	16·9	64·5	72·9	51	43
Aug. 5	8·4	9·9	11·7	14·7	66·0	63·8	79	46
„ 20	3·3	5·9	—	8·5	59·5	67·1	140	70
Sept. 5	2·4	5·0	9·8	7·4	—	64·0*	(262)	(145)
„ 11	3·4	3·5	4·4	—	64·0*	—	Spent	Spent

* Corrected as described on p. 2030.

From the figures in Table IX it is seen that there is a steady loss of cholesterol from the liver and intestine, and a rapid gain by the gonad of that substance, while the figure for the muscle remains constant. Further, at every stage of maturation the milt contains considerably more cholesterol than the roe.

It seems to us that these figures provide evidence that the fish is synthesising cholesterol. In view of the uncertainty which exists as to the last figure for the gonad-cholesterol on September 5, as discussed on p. 2030, it may be wise to consider that of August 20, which must be very considerably less than the true value for September 5. In the period July 24–August 20 the gonads have gained 56 mg. of cholesterol. In the same period the liver on

the average has lost about 9 mg. and the intestine 6·3 mg., a total of about 15 mg. Bearing in mind the fact that the fish is not feeding at this time, this suggests a synthesis of cholesterol by the herring of about 40 mg. in 6 weeks. The possibility that there had been some mobilisation of sterol from the heads of the fish was not investigated at the time, but has been determined since on heads of fish taken in September 1932. The heads of 8 mature and 8 spent 5-year old fish, for the collection of which we wish to thank Mr W. C. Smith, were saponified in the usual manner and the unsaponifiable fraction was prepared, and the amount of cholesterol present determined. The average figures for the heads of these fish were as follows:

	Wt. of unsap. fraction mg.	Wt. of cholesterol mg.
Mature fish	63	47·1 per head
Spent fish	54·5	41·2

The differences between the figures for the mature and spent fish are insignificant, and hence we incline to the view that the fish is synthesising sterol during the later stages at least of the maturation period.

Summary.

1. A quantitative study has been made of the fatty acids and cholesterol in the different tissues of the Manx herring from the time when the gonads are empty, through the stages of complete maturation to the spawning of the fish about 12 weeks later. Age, weight, length and sex have been considered.

2. The percentages of fatty acids in the liver and "mesentery" increase for 3 weeks to a maximum (15 and 39 % respectively) and then fall to about 1 % by the time of spawning. The maximum in the muscles, 17·4 %, occurs rather later (about 5 weeks) and then progressively falls, approaching the initial value. Meanwhile the rapidly growing ovaries and testes show varying percentages throughout.

3. The percentage of cholesterol in the gonads, however, continuously rises to a maximum with maturation. That present in the liver and "mesenteric" fat disappears at a far slower rate than do the fatty acids of these tissues, while the percentage in the muscle remains approximately constant throughout.

4. Because, with tissues changing so rapidly in weight, the percentage values give no idea of the absolute changes involved, an attempt has been made to interpret the data along these lines. The probability of the assumptions necessary for this purpose is discussed. The balance-sheet so constructed shows that the loss of fatty acid from the time when the maximum values obtain to the time of maturation are: liver 0·25 g., "mesentery" 2·65 g., muscle 3·76 g. Meanwhile the gonads have gained 0·98 g. The significance of these various changes is discussed.

5. It shows further that, in the last stages of maturation at least, the fish is synthesising cholesterol.

6. The degree of unsaturation of the fatty acids of the ovaries and testes rises steadily to a maximum before the fish spawns, when the iodine value has risen from 137 to 200. The iodine values of the acids of the other tissues have however remained constant, the values being for liver 128, mesentery 108, muscle 118. The possible significance of the finding is discussed.

REFERENCES.

Bruce (1924). *Biochem. J.* **18**, 469.
Johnstone (1915). 23*rd Ann. Rep. Lancashire Sea Fish. Lab.* 154.
—— (1918). 26*th Ann. Rep. Lancashire Sea Fish. Lab.* 13.
—— (1919). 27*th Ann. Rep. Lancashire Sea Fish. Lab.* 36.
—— (1920). 28*th Ann. Rep. Lancashire Sea Fish. Lab.* 16.
Lea (1910). *Cons. Int. Expl. Mer., Public de Circ.*, No. 53, 7.
Milroy (1906). 24*th Ann. Rep. Fishery Bd. for Scotland*, Pt. III, 83.
—— (1907). 25*th Ann. Rep. Fishery Bd. for Scotland*, Pt. III, 197.
—— (1908). *Biochem. J.* **3**, 366.
Rosenmund and Kuhnhenn (1923). *Z. Untersuch. Nahr. Genussm.* **46**, 154.
Saby (1932). 40*th Ann. Rep. Lancashire Sea Fish. Lab.* 139.

CCXLII. VITAMIN B_4.

By HAROLD BARNES, JOHN RICHARD PERCIVAL O'BRIEN and VERA READER[1].

From the Department of Biochemistry, Oxford.

(*Received October 29th, 1932.*)

In earlier publications [Reader, 1929; 1930, 1, 2] the evidence has been presented for the conclusion that crude watery or alcoholic extracts of yeast contain two heat- and alkali-labile factors necessary for the nutrition of the rat; in the literature these two factors are referred to as vitamin B_1 (the antineuritic vitamin) and vitamin B_4. A method of assay of vitamin B_4 has been described in detail by Reader [1930, 2] and has since been used with success by Halliday [1932]. As an appendix to the above paper [1930, 2] Reader described a method for preparing vitamin B_4 concentrates free from vitamin B_1, making use of the mercuric sulphate fraction obtained in the Kinnersley and Peters process for vitamin B_1. A stable concentrate (activity 0·6 mg. per rat day dose) was obtained, but further purification seemed impossible without loss of stability; consequently the method was abandoned for one in which the vitamin B_4 was removed from the yeast extracts by adsorption on charcoal at p_H 1·0 [*vide* Peters, 1931].

From these activated charcoals we have now prepared a white crystalline material of high vitamin B_4 activity and of apparently constant chemical composition. Essential steps in this new process are fractionation with sodium phosphotungstate [*cf.* Peters, 1930] and liberation of an actual base by hydrolysis of a base-pentose compound. We hesitate to describe our compound as vitamin B_4 itself, as we have certain indirect evidence as yet unpublished (Kinnersley and Peters), that a preparation of vitamin B_4 of higher activity can be obtained. Our crystals, stored in a desiccator over calcium chloride, have remained stable for 6 months and so form a useful standard for further work on vitamin B_4.

Experimental.

Testing. Throughout this work the adult rat curative method of assay [Reader, 1930, 2] was used to follow the vitamin B_4 activity. As previously mentioned our stock colony has been rigorously inbred in order to make the variation in response between the individual animals as small as possible. At least 5 animals have been used for testing the extracts after each stage in

[1] A preliminary account of this work has previously been published [Barnes, O'Brien and Reader, 1932]. Although the research is still incomplete we have decided to publish a detailed account, as one of us (V. R.) is unable to continue the work.

each preparation. After the phosphotungstate stage not more than 2 in any 20 animals gave results outside a $\pm$ 20 % range.

Plate IX shows (A) the condition of an animal when ready for test, and (B) the state of the same animal after 1 week's treatment with the active substance. When in condition (A), the animal is unable to walk without falling (usually to the left side), and cannot control the action of its head sufficiently to drink from the usual vertical bottle. Lack of co-ordination is the most marked symptom, but is frequently accompanied by a noticeable dilation of the capillaries between the digits of the paws.

Purification of active material. The preparation of vitamin B_4-active charcoals from yeast has been described elsewhere [Peters, 1931]. In this paper the technique used in preparing the crystalline material from these charcoals is described. The quantities of materials stated correspond to those required for the charcoals from one batch of 50 kg. of the original yeast. The p_H values were determined by the colorimetric method and are only approximate.

(*a*) *Extraction.* Immediately after the adsorption process, the charcoals were filtered on Büchner funnels, washed with dilute sulphuric acid (p_H 1) and then covered with 50 % ethyl alcohol to which was added sufficient concentrated hydrochloric acid to bring the fluid in contact with the charcoal to p_H 1·0. Four successive extractions, each with 800 cc. of fluid, were carried out by warming on the water-bath to 70° and filtering at the pump whilst hot. The total extraction fluids were mixed, sodium hydroxide was added to bring the p_H to 3·0, and the whole distilled *in vacuo* to remove alcohol. The residue, having a volume of approximately 1500 cc., was allowed to stand in cold store overnight.

(*b*) *Mercuric sulphate treatment.* The extract was again adjusted to p_H 3·0, and 350 cc. of Denigès's mercuric sulphate reagent added. After standing for 1 hour, the fluid was filtered, the precipitate being discarded: excess sulphate was removed from the filtrate by addition of hot saturated solution of baryta, until the p_H was 2·0. After standing another 2 hours, the fluid was filtered, treated with sodium hydroxide to bring it to p_H 4·0, and then with H_2S for 4 hours to remove mercury. After again filtering, the excess H_2S was removed *in vacuo*, the temperature not being allowed to exceed 30°, to prevent appreciable reduction of volume. The fluid, about 2000 cc., was allowed to stand in cold store overnight.

This treatment removes a certain amount of material without diminishing the activity of the extract. Such a result is in contrast with earlier experiments [*vide* Reader, 1930, 2] in which the active substance itself was precipitated by the mercuric sulphate reagent. There can be little doubt that the adsorption on charcoal, introduced into the present technique, is responsible for this altered behaviour.

(*c*) *Fractional precipitation with sodium phosphotungstate.* Several preliminary experiments were conducted, in which the merits of the 1:18 and the 1:24 phosphotungstates for fractional precipitation of the vitamin were

(A) Animal suffering from deficiency of vitamin B_4.

(B) Same animal after one week's treatment with active material (0·01 mg. daily).

(C) Crystals with vitamin B_4 activity. ×100.

compared. A typical result is shown in Table I. To each of two flasks, each containing 20 cc. of the fluid (50 rat doses), 1 cc. of the appropriate phosphotungstate was added, and the p_H gradually changed from 6 to 1, precipitates being removed at the points indicated in Table I. Definite zones of precipitation were observed [*cf.* Kinnersley and Peters, 1930].

Table I.

	p_H at which precipitate removed	Approx. no. of vitamin doses recovered
Sodium salt of 1:18 acid	4·0	5
,,	2·5	10
,,	1·0	10
Sodium salt of 1:24 acid	4·0	0
,,	2·5	10
,,	1·0	40

From such results as are shown in Table I it was concluded that a more specific precipitation occurred with the 1:24 salt and that the maximum point for the vitamin was between p_H 2·5 and 1·0. Hence it was decided to use the 1:24 salt (prepared by the method of Wu [1920]) for all further work. Immediately before use 10 g. of the acid were dissolved in 50 cc. of distilled water, 20 % sodium hydroxide was added to bring the p_H to 6·0, and the whole made up to 100 cc.

The fluid from the mercury stage (*b*) was then brought up to p_H 6·0 and an excess of the 10 % phosphotungstate solution (about 50 cc.) added. 5 % sulphuric acid was gradually added to bring the whole to p_H 3·0. It was then allowed to stand in the cold store overnight. Next morning it was filtered and the precipitate discarded. 5 % sulphuric acid was again added to bring the filtrate to p_H 1·0 and the mixture allowed to stand in the cold store overnight. After filtration at the pump, the precipitate, which was straw-coloured, contained the activity.

(*d*) *Recrystallisation of the active phosphotungstate.* The above precipitate was washed through the filter-paper with the smallest possible quantity of hot 50 % ethyl alcohol (about 100 cc.) and allowed to stand in a cool place for 24 hours. Any precipitate which was not soluble in the hot alcohol was discarded.

(*e*) *Removal of phosphotungstic acid.* Next day the yellow deposit (usually, in the main, crystalline) was filtered from the alcohol, dissolved in 50 % acetone, and cold saturated baryta added until the solution turned phenolphthalein pink. It was then shaken well for 5 minutes and filtered as rapidly as possible. A few drops of dilute sulphuric acid were added to the filtrate immediately to remove any excess barium and to make the solution acid again (p_H 3). This filtrate, volume about 250 cc., and containing 30–40 % acetone, should contain 10–15 rat day doses of vitamin B_4 per cc. Test doses were always given to the animals before proceeding further. The acetone

present in 1/10 cc. of the solution was enjoyed by the animals and did not appear to do them any harm. The small deposit of barium sulphate was removed by decantation or filtration immediately before use. In all 19 lots, each representing 50 kg. of yeast were brought to this stage. All were free from vitamin B_1 and from the streptothrix factor [Reader, 1928] and have remained stable for many months.

(*f*) *Hydrolysis.* Acetone was removed *in vacuo* and the fluid concentrated to 50 cc. 5 cc. of concentrated hydrochloric acid were added and the whole was heated on the water-bath for 1–2 hours, or until a positive orcinol reaction for pentose was obtained. The time taken for this hydrolysis varied from 1 to 3 hours in the 17 lots tried, but the average time was 1 hour. It is useless to proceed to the next stage unless a positive orcinol reaction is obtained.

(*g*) *Crystallisation.* The 50 cc. fluid from the above were concentrated to 10 cc. on the water-bath and 20 cc. of acetone added. If any precipitate formed it was removed by filtration and discarded. (Last traces of barium sulphate and chloride were thus removed.) Then 70 cc. of ether were cautiously added and, after gentle stirring, the mixture was allowed to stand on ice for 12 hours or until crystals appeared. On two occasions the crystals appeared within 2 minutes from the time of addition of the ether. The product was recrystallised from a 1 part water–5 parts acetone–50 parts ether mixture. A photomicrograph of the final product is shown in Plate IX (C).

Results.

During the last year about a ton of yeast has been worked through the stages described above. The results from 12 successive lots, each of 50 kg., are recorded in Table II. The numbers represent the total number of rat day doses (curative) in the extracts.

Table II.

Lot	Extract from charcoal	After mercury	After phospho-tungstic	After hydrolysis	Remarks
1	5000	5500	4000	4000	No pentose and no crystals
2	6000	5500	3000	Nil	—
3	6000	6000	5000	4000	30 mg. crystals
4	4500	4000	4000	3500	28 mg. crystals
5	5000	4500	4500	3000	15 mg. crystals
6	6000	5500	5500	3000	No pentose and no crystals
7	5500	5000	5000	4000	20 mg. crystals
8	4500	4200	4000	2500	10 mg. crystals
9	4800	4500	4500	Nil	—
10	6000	6000	5500	5000	5 mg. crystals
11	6000	5000	5500	4500	Nil
12	6500	6000	5000	1000	Nil

It will be seen that the method worked fairly constantly without great loss of activity up to the end of section (*e*), *i.e.* liberation from phosphotungstic acid: but that for the hydrolysis and crystallisation stages the conditions are not yet well defined.

The improvement in purity as the preparation proceeds is shown in Table III. Lots (3), (4) and (5) from Table II are quoted as illustrations.

Table III.

Stage	Activity (organic solid weight required per unit dose (± 20 % variation)). mg. Lot 3	Lot 4	Lot 5
Extract from charcoal	10	9	10
After mercury	5	4	6
After phosphotungstic	0·5–0·2	0·5–0·3	1·5–0·5
Crystalline material	0·010	0·012	0·010

Analysis. Samples of crystals from lots 3 and 5 have been analysed by Dr Ing. Schoeller of Berlin. His findings are:

	C %	H %	N %	Cl %
Lot 3	35·5	3·5	38·03	21·6
Lot 5	34·8	3·4	37·70	22·2

The above figures were obtained after drying the substance at 80° in high vacuum over P_2O_5. The water content was 5·2 % and 5·4 % for the two samples respectively. The empirical formula is probably $C_4H_4N_4.HCl, \frac{1}{2}H_2O$.

Properties. Hydrochloride of a base: sublimes at 220°: very soluble in water: insoluble in absolute alcohol, ether or acetone: forms a crystalline picrate which melts at 278°: is adsorbed on charcoal at p_H 2·0: precipitated by sodium phosphotungstate (1:24) from a 1 in 500 solution at p_H 4·8: gives negative results for the following colour reactions: Pauly, Fehling, Millon, α-naphthol, and Tollens.

Stability to acid. The final product, a base forming salts with acids, is stable to acid treatment, even boiling with 20 % HCl for an hour. At first sight this does not harmonise with earlier results of Reader [1929], in which it was shown that after boiling a watery extract of yeast or marmite for an hour with 5 % HCl, a 50 % destruction of the vitamin had occurred. Again after liberation from phosphotungstic acid, the activity of the solution remains stable so long as acetone or alcohol is present but diminishes on storage, even at 0° in an aqueous solution. It would appear therefore that the stability of the product towards acid is variable and is influenced by the degree of purification achieved.

Stability to alkali. At all stages in the preparation the vitamin B_4 activity is lost on boiling with alkali. The final crystalline material is inactivated by heating on the water-bath with 10 % sodium hydroxide for 10 minutes. So far none of the crystalline base has ever been recovered after this treatment, but this may be due to the small amounts of material handled.

SUMMARY.

An account is given of an attempt to isolate vitamin B_4 from yeast. An unknown base, with a high and apparently constant vitamin content, has been isolated in the form of colourless crystals. Essential steps in its preparation are fractional precipitation with phosphotungstate, and removal of a pentose group from the molecule by acid-hydrolysis. Analyses suggest the formula $C_4H_4N_4.HCl, \frac{1}{2}H_2O$. Further work is required before it can be claimed that the product is identical with the vitamin.

In conclusion we wish to thank Prof. R. A. Peters for his advice and encouragement throughout the research, and to acknowledge gratefully a personal grant from the Medical Research Council to one of us (V. R.); also to thank Miss M. Kempson for her assistance and care with the animals.

REFERENCES.

Barnes, O'Brien and Reader (1932). *J. Physiol. Proc.* **76**, 8 *P*.
Halliday (1932). *J. Biol. Chem.* **96**, 479.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 1856.
Peters (1930). *Biochem. J.* **24**, 1852.
—— (1931). *Report Chem. Sec. Brit. Assoc. Meeting*, p. 131.
Reader (1928). *Biochem. J.* **22**, 434.
—— (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.
Wu (1920). *J. Biol. Chem.* **43**, 189.

CCXLIII. STUDIES IN THE SULPHUR METABOLISM OF THE DOG.

XI. THE METABOLISM OF METHIONINE AND RELATED SULPHIDES.

By NORMAN WINGATE PIRIE.

From the Biochemical Laboratory, Cambridge.

(*Received October 30th, 1932.*)

It has long been known that only a fraction of the sulphur in many proteins is present in the form of cystine [Johnson, 1911; Harris, 1923] and it has been known for several years that a part of the remaining sulphur is in the form of methionine. I have already shown that methionine is the principal sulphur-containing amino-acid in caseinogen; this is probably also true in the case of egg-white, for I have isolated, by the same method, 2·5 % of methionine from it. Baernstein [1932], having assumed that proteins contain no methoxyl compounds and no methylthiol compound other than methionine, has published values for the methionine content of a large number of proteins. The validity of his assumptions is doubtful but the values obtained, when taken in conjunction with the cystine contents, enable him to account for approximately 100 % of the sulphur in the proteins used. In most cases the greater part of the sulphur is found to be in the form of methionine. It appears therefore that methionine is generally the principal sulphur-containing constituent of proteins other than scleroproteins and is quantitatively the most important sulphur compound in an ordinary diet. Jackson and Block [1931] and Weichselbaum, Weichselbaum and Stewart [1932] have shown that methionine will supplement a cystine-low diet, but the problem of preparing a diet that is free from either cystine or methionine is still unsolved.

Mueller [1924] showed that, in man, methionine was readily oxidised to sulphate; his experiments were necessarily short and one cannot determine from them the completeness of oxidation of the amino-acid. The present work is an extension of that of Mueller. Dogs have been used and methionine has been compared with cystine on the one hand and with some other sulphides, namely *S*-benzylcysteine, *S*-ethylcysteine and *S*-methylcysteine, on the other.

Methionine is readily oxidised by the dog. The results of three experiments are shown in the figure and are analysed in the table on p. 2042.

The obvious similarity between the metabolic behaviours of methionine and cystine is interesting in view of their widely different chemical structures.

S-Ethylcysteine [Clarke and Inouye, 1931], although closely related chemically to methionine is not oxidised to any appreciable extent by the dog. It is

Substance	Dog	mg. S given	% excreted as sulphate			% excreted as neutral S			% of dose accounted for
			1st day	2nd day	Total	1st day	2nd day	Total	
Methionine	M	320	46	13	59	10	10	20	79
,,	P	320	47	19	66	11	9	20	86
,,	P	320	58	14	72	9	0	9	81
Cystine	M	320	49	11	60	6	4	10	70
,,	P	320	60	11	71	10	3	13	84

These results may be summarised thus.

	% excreted as sulphate in 2 days	% excreted as neutral sulphur in 2 days
Methionine	66	16
Cystine	66	11
Cystine and cysteine [Hele and Pirie, 1931]	70	4

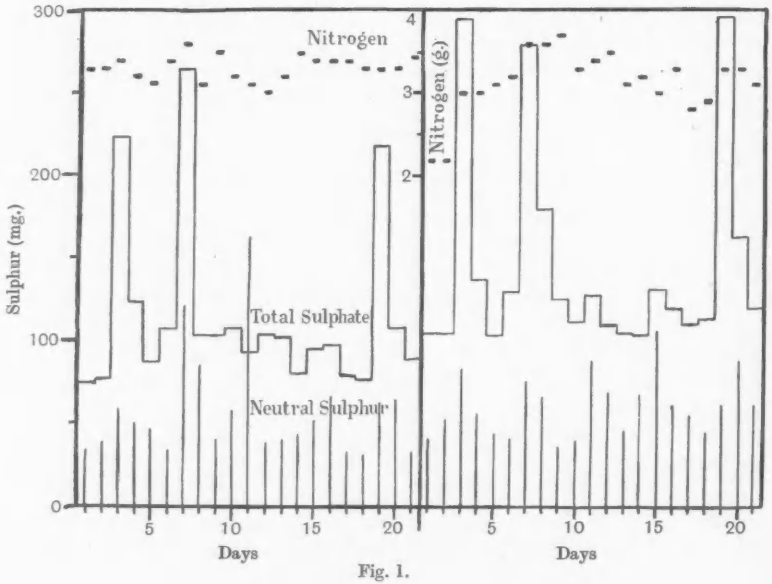


Fig. 1.

MAUD.

Day 3. 1·2 g. cystine orally (320 mg. S)
,, 7. 1·46 g. cysteine HCl subcut. (300 mg. S)
,, 11. 2·11 g. *S*-benzylcysteine orally (320 mg. S)
,, 15. 0·84 g. *S*-ethylcysteine orally (179 mg. S)
,, 19. 1·49 g. methionine orally (320 mg. S)

PATSY.

Day 3. 1·2 g. cysteine orally (320 mg. S)
,, 7. 1·49 g. methionine orally (320 mg. S)
,, 11. 1·49 g. *S*-ethylcysteine orally (320 mg. S)
,, 15. 1·09 g. *S*-benzylcysteine orally (164 mg. S)
,, 19. 1·49 g. methionine orally (320 mg. S)

slightly toxic; both dogs were somewhat lethargic for an hour or two after the dose while one of them (Patsy) vomited a little 11 hours after the larger dose (1·49 g.). The metabolic upset must have been slight since there was no marked effect on the excretion of nitrogen.

No very definite conclusions could be drawn concerning the oxidation of *S*-methylcysteine[1]. Both dogs vomited when given 0·8 g. orally while Maud was made seriously ill by 0·5 g. given subcutaneously, in this case there was also a trace of blood in the urine. Smaller doses than these would contain so little sulphur that it would be difficult to tell whether much had been oxidised or not. There was a definite rise in the total sulphate excretion on both the occasions when the substance was fed, corresponding to 25 % of the dose in the case of Maud and 40 % in the case of Patsy. In each case the urine was contaminated and the effect of the general disturbance on the sulphur metabolism is unknown. It seems to be fairly certain however that *S*-methylcysteine is more readily oxidised than *S*-ethylcysteine but much less readily oxidised than its homologue methionine. It would be interesting to study the behaviour of *homo*methionine and *S*-ethyl*homo*cysteine, *i.e.*

$CH_3SCH_2CH_2CH_2CH(NH_2)COOH$ and $CH_3CH_2SCH_2CH_2CH(NH_2)COOH$.

Differences such as those already mentioned in the metabolism of closely related substances emphasise the need for extreme caution in drawing conclusions, such as those of Sherwin, Shiple and Rose [1927], from the metabolic behaviour of derivatives of cystine or cysteine of unrelated character.

S-Benzylcysteine was prepared according to Suter [1895]. When given by mouth (1·09 g.) it was oxidised to a slight and probably not significant extent by one dog while the other dog did not oxidise a larger dose (2·11 g.) at all. These results contrast with those of Sherwin, Shiple and Rose [1927] who found that the rabbit could oxidise 40 % of the sulphur to sulphate after oral administration of *S*-benzylcysteine. Differences of this sort may easily be due, as Lewis [1924] has pointed out to differences in the intestinal flora of different species. It is noticeable that with *S*-benzylcysteine, as with *S*-ethylcysteine, only about a third of the extra sulphur fed is accounted for in the total sulphur value of the urine. This low recovery is not due to the deficiencies of the method of estimation used [Pirie, 1932, and Appendix to this paper] for control experiments show that 80–90 % of the sulphur in *S*-benzylcysteine or *S*-ethylcysteine added to urine, is converted into sulphate by this method of incineration. There was no change in the excretion of ethereal sulphate after feeding *S*-benzylcysteine. It was noticed that both *S*-benzylcysteine and *S*-ethylcysteine caused a transient diuresis, the volume of urine passed in the course of 24 hours was not increased but in all cases more than usual was passed during the first 12 hours.

Feeding experiments have also been carried out with a number of

[1] The preparation of *S*-methylcysteine has not apparently been described before. It was made by the standard technique with dimethyl sulphate and cysteine hydrochloride. The preparation will be described shortly.

sulphoxides; these will be described fully in a later paper. Preliminary experiments show that methionine sulphoxide is as readily oxidised as methionine whereas the sulphoxides of *S*-ethyl- and *S*-benzylcysteine are completely resistant to oxidation by the dog.

Experimental.

Two bitches, Maud and Patsy, were used. The former has not been used for metabolic work before while the latter is a veteran. The course of the experiments was exactly the same as in the earlier papers of this series [Hele, 1924] and the dogs received the following diets:

	Lean meat g.	Sugar g.	Margarine g.	Milk cc.	Water cc.	Agar g.
Maud (7·3 kg.)	90	60	20	160	100	5
Patsy (7·7 kg.)	70	50	20	160	100	5

There was no alteration in the dogs' weights during the experiments. Agar has recently been added to our standard dog diet since some unpublished work, carried out in collaboration with Hele, has shown that it increases the absorption of some inorganic constituents of the diet.

Total sulphate estimations were carried out by Folin's [1905] method and total sulphur by the method described in the Appendix to this paper. Inorganic sulphate estimations, by the benzidine method, were only made after feeding benzylcysteine; they showed that there had been no change in the excretion of ethereal sulphate.

Summary.

Methionine is oxidised by the dog to the same extent as cystine.

S-ethylcysteine and *S*-benzylcysteine are not appreciably oxidised.

S-methylcysteine is oxidised to some extent but is too toxic to allow an accurate estimate of its oxidation to be made.

I am grateful to Sir F. G. Hopkins for his interest in this work. The expenses of the research were largely covered by a grant to Dr Hele from the Royal Society.

APPENDIX.

Determination of total sulphur in dog's urine.

The estimation of total sulphur in urine is much the most laborious part of the work entailed in studying sulphur metabolism. We have hitherto used Folin's [1905] method; this involves fusion with sodium peroxide and it is the fusion that is most troublesome. The subsequent precipitation of barium sulphate and weighing is, we think, unavoidable with dog urine. Those methods which employ a titration (*e.g.* using benzidine [Fiske, 1921] or barium chromate [Morgulis and Hemphill, 1932]) involve the preliminary removal of phosphate from the urine. I have found that the published methods of doing this, although no doubt admirable when used with human urine, remove part of the neutral sulphur from dog's urine.

The incineration technique now adopted is based on that of Lematte, Boinot and Kahane [1927].

The oxidising agent consists of 3 volumes of concentrated nitric acid and 1 volume of 60 % perchloric acid, 3 volumes of this mixture are then mixed with 1 volume of the same mixture saturated with copper nitrate. The reagent is perfectly stable.

5 cc. of filtered urine and 5 cc. of the acid mixture are heated in a 6 × 1 in. monax test-tube which is two-thirds immersed in an air-bath at about 200°. The temperature is allowed to rise slowly and after 2–3 hours the contents of the tube are dry. It is removed from the bath, allowed to cool for a minute, and 1 cc. of the acid mixture added. After a further 2–3 hours in the bath at a temperature up to 280° the contents will be black or a dirty green, they should not still be blue. The tube is now held in crucible tongs while being heated all over for a few seconds in a large, but not very hot, Bunsen flame. If the heating in the air-bath has been adequate there will only be a few flashes, due to residual perchlorate, in the upper parts of the tube. When cool 2 cc. of *N* hydrochloric acid are added and the tube is replaced in the air-bath while latter is cooling. Boiling water is added till the tube is two-thirds full followed by 5 cc. of the 5 % barium chloride solution. The barium sulphate may be filtered off and weighed in the usual way after 2–4 hours. As a rule the quantity of barium sulphate weighed is of the order of 20 mg., the crucibles must therefore be packed carefully. I have found no difficulty in getting duplicates consistently agreeing to within 0·3 mg.; since the incineration and precipitation are carried out in the same vessel loss can only occur during the filtration. The same Gooch crucible can safely be used 4–5 times without re-packing with these small amounts of sulphate.

The air-bath used consists of a heavy iron saucepan with an inch of sand in it, an unglazed earthenware plate which is 1 inch less in diameter than the pot is supported on legs over the sand. The tubes and a thermometer pass through holes in an asbestos sheet cover and rest on the plate. The whole is heated by a large burner. Twelve tubes can conveniently be heated simultaneously.

This method has been checked on a number of different dog urines against my own estimations and those of Hele with the peroxide method. The differences were within the experimental error.

When used with materials having a low salt content, amino-acid fractions from a protein for example, the incineration can be carried through in an hour and, if very accurate results are not required, the estimation may be finished by the benzidine method.

REFERENCES.

Baernstein (1932). *J. Biol. Chem.* **97**, 669.
Clarke and Inouye (1931). *J. Biol. Chem.* **94**, 548.
Fiske (1921). *J. Biol. Chem.* **47**, 59.
Folin (1905). *J. Biol. Chem.* **1**, 131.
Harris (1923). *Proc. Roy. Soc. Lond.* B **94**, 426.
Hele (1924). *Biochem. J.* **18**, 586.
—— and Pirie (1931). *Biochem. J.* **25**, 1095.
Jackson and Block (1931). *Science*, **74**, 414.
Johnson (1911). *J. Biol. Chem.* **9**, 437.
Lematte, Boinot and Kahane (1927). *J. Pharm. Chim.* **5**, 325.
Lewis (1924). *Physiol. Rev.* **4**, 408.
Morgulis and Hemphill (1932). *J. Biol. Chem.* **96**, 576.
Mueller (1924). *J. Biol. Chem.* **58**, 373.
Pirie (1932). *Biochem. J.* **26**, 1270.
Sherwin, Shiple and Rose (1927). *J. Biol. Chem.* **73**, 667.
Suter (1895). *Z. physiol. Chem.* **20**, 562.
Weichselbaum, Weichselbaum and Stewart (1932). *Nature*, **129**, 795.

CCXLIV. THE COLORIMETRIC DETERMINATION OF PHOSPHORUS IN THE PRESENCE OF INTERFERING SUBSTANCES.

By DANIEL RICHARD DAVIES and WALTER CULE DAVIES.

From the Biochemical Laboratory, Cardiff City Mental Hospital, and the Tatem Laboratories, University College, Cardiff.

(*Received October 31st, 1932.*)

Recently, the present authors [Davies and Davies, 1931] described a method for the elementary analysis of phosphorus in pure organic compounds, by a modification of the Briggs-Bell-Doisy method. It was observed during the course of the above investigation that it was necessary to standardise the conditions exactly, in order to ensure accuracy. At the same time, one of us was working with Dr J. H. Quastel, in the Biochemical Laboratory of the Cardiff City Mental Hospital, upon the presence of phosphatases in brain tissue. It was then noted that sufficiently large quantities of potassium pyrophosphate prevented the formation of the characteristic blue acid, due to the reduction of the yellow phosphomolybdic acid, resulting from the interaction between molybdic and orthophosphoric acids.

Fiske and Subbarow [1925], Vásárhelyi [1929], and King [1932] have shown that 1:2:4-aminonaphtholsulphonic acid, used in the place of quinol for the reduction of the yellow phosphomolybdic acid, diminishes the effect of many substances, such as sodium chloride, sodium nitrate and ammonium sulphate, all of which hinder the formation of the blue colour. Denis and Meysenbug [1922] found that in certain cases the original Bell and Doisy method gave anomalous results with both citrated and oxalated plasmas, and recommended that it was desirable to restrict the amount of anticoagulant when estimating the inorganic phosphate of blood. Whilst this paper was being written, Tschopp and Tschopp [1932] reported that a quantitative estimation of the orthophosphate ion by their modification of the colorimetric method was only possible when there were less than 30 mg. of ammonium oxalate, or 50 mg. of sodium citrate or 50 mg. of tartaric acid per 0·04 mg. of phosphorus present. They also discussed the effects of ferrous, ferric, cuprous and manganous ions and also of silicates and arsenates.

In the present work it has been found that a number of organic acids and their salts, which may often occur together with phosphorus-containing compounds in biological material, or may be added during biochemical manipulations, can, if present in sufficiently large quantities, entirely prevent the formation of the blue colour. Hence it has been felt necessary to investigate

the maximum quantity of the interfering substance with which it is possible to estimate orthophosphate satisfactorily by the colorimetric method. When the amount of interfering substance is sufficiently large to affect the procedure, we have sought to modify our method to enable us to carry out the estimation accurately.

The effect of sodium and potassium pyrophosphate.

Table I illustrates the effect of increasing quantities of potassium pyrophosphate upon the estimation of 0·08 mg. of phosphorus present as potassium dihydrogen phosphate.

Table I. *The effect of potassium pyrophosphate.*

Tube No.	Mg. potassium pyrophosphate	Mg. phosphorus recovered	Percentage recovered	After 12 hours. Mg. phosphorus recovered
1	0	0·08	100	—
2	12·5	0·083	104	—
3	25	0·086	107·5	—
4	30	0·093	116	—
5	35	0·083	104	—
6	37·5	0·076	95	—
7	40	0·071	89	—
8	45	0·048	60	—
9	50	0·020	25	0·25
10	62·5	0·0	0	0·16
11	75	0·0	0	0·06
12	87·5	0·0	0	0·0
13	100	0·0	0	0·0

To each tube were added 1 cc. of potassium dihydrogen phosphate solution containing 0·08 mg. of phosphorus, increasing quantities of potassium pyrophosphate, 2 cc. of 5·5 *N* sulphuric acid, 2 cc. of 5 % ammonium molybdate, distilled water to about 12 cc. and 1 cc. of reducing agent (15 % sodium sulphite and 0·5 % quinol). The final volume was made up to 15 cc. with water and the contents of the tubes were allowed to stand for 30 minutes. At the end of this period the colour intensity in each tube was matched against an appropriate standard in a Klett top-reader colorimeter. It was so arranged that the colours of the standard and test solutions were almost identical.

Until a concentration of 2·5 mg. (No. 6) of potassium pyrophosphate per 1 cc. of reaction mixture was reached a more than theoretical recovery of orthophosphate was obtained. This was probably due to hydrolysis of the pyrophosphate in the acid solution producing orthophosphate, and perhaps to some extent slight contamination of our pyrophosphate sample. It should be noted, for example, in No. 4 in which the maximum increase in recovery of phosphorus was obtained, that this represented less than 0·3 % of the phosphorus content of the pyrophosphate present. Above concentrations of 2·5 mg. per cc. of reaction mixture the colour became less intense and finally disappeared at concentrations above 4 mg. The last column of Table I shows the effect of allowing the tubes to stand for 12 hours. This series gives further information concerning the nature of the hydrolysis of the pyrophosphate (see p. 2053).

The graphs, Fig. 1, show that the amount of sodium pyrophosphate necessary just to prevent the formation of the blue colour is proportional to the concentration of the ammonium molybdate reagent. The three series of experiments here figured only differ from those given in Table I in the quantities of ammonium molybdate added. Thus, using 1, 2 or 3 cc. of molybdate complete absence of colour commences when 2, 4 or 6 mg., respectively, of sodium pyrophosphate per cc. of the reaction mixture are added. These results indicate that the mechanism of the inhibition is one of complex salt formation between the alkali pyrophosphate and the ammonium molybdate. At con-

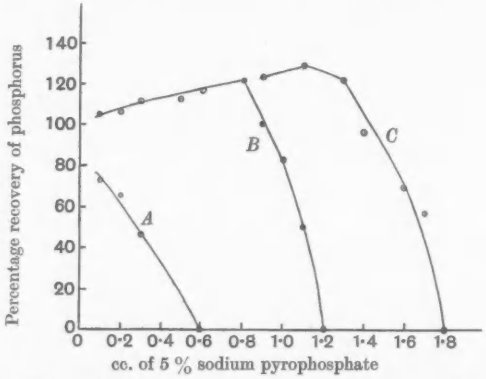


Fig. 1. Effect of variation of the amount of ammonium molybdate on the recovery of orthophosphate (0·08 mg. of phosphorus) in the presence of increasing amounts of sodium pyrophosphate. *A*, 1 cc., *B*, 2 cc., *C*, 3 cc. of 5 % ammonium molybdate.

centrations where complete absence of blue colour occurs, there is sufficient pyrophosphate present to use up the whole of the molybdate, so that no phosphomolybdate can be formed for ultimate reduction to the blue complex. The addition of more molybdate results in the reappearance of the blue colour.

Table II shows the effect of two different reducing agents and of increasing the hydrogen ion concentration on the recovery of orthophosphate from solutions containing increasing amounts of sodium pyrophosphate.

The tubes were made up as described for Table I, except for the acidities, which are given in the table, and the reducing agents, which are given below.

Reducing agent A. 1 cc. of quinol reagent. The colours were compared with the standards after 30 minutes with this reagent.

Reducing agent B. 0·5 cc. of 1:2:4-aminonaphtholsulphonic acid reagent (0·5 g. of the acid, 30 g. of sodium bisulphite and 6 g. of sodium sulphite made up to 250 cc. [King, 1932]). The colours were compared with the standards according to the directions given at the head of the table.

Under the standard conditions of acidity (2 cc. of 5·5 *N* H_2SO_4) it will be seen that with 1:2:4-aminonaphtholsulphonic acid as reducing agent rather more pyrophosphate was required to cause complete inhibition than with

Table II. *The effect of different reducing agents and of increased acidity.*

		Percentage recovery of phosphorus				
		Reducer A		Reducer B		
		Acidity in cc. 5·5 *N* H_2SO_4				
Tube No.	Mg. sodium pyrophosphate	2 cc.	4 cc.	2 cc. Read after 5 mins.	2 cc. After 20 mins.	4 cc. After 5 mins.
1	5	106	<50	—	—	<50
2	10	106·5	|	100	—	|
3	15	112	Gradual decrease	—	115	Gradual decrease
4	20	—	with no sharp	73	—	with no sharp
5	25	112·5	break	—	116	break
6	30	117	|	67	—	|
7	35	—	|	—	116	|
8	50	83·5	|	—	114	0
9	55	—	0	—	—	
10	60	0		—	—	
11	65	—		—	96	
12	75	—		—	<50	
13	85	—		0	—	
14	90	—		—	0	

quinol. The difference might possibly be ascribed to the greater sensitivity of the naphthol derivative as a reducing agent. Because of the increased time for which estimations with quinol have to be allowed to stand before comparison the hydrolytic process becomes apparent. This is also shown if the colours developed with reagent B are allowed to stand and then matched.

When the acidity is increased the weakening in the blue colour occurs with much smaller concentrations of pyrophosphate, but the point at which colour completely disappears is almost the same using either of the reducing agents. A similar decrease in the intensity of the blue colour is seen even in the absence of pyrophosphate when the acidity is increased, so that no importance can be attached to the above result.

It is only possible to estimate orthophosphate accurately by the colorimetric method when there are less than 10 mg. of alkali pyrophosphate present per 0·1 g. of ammonium molybdate. Also the reducing agent used should be one with which maximum colour intensity is reached rapidly (*e.g.* 1:2:4-aminonaphtholsulphonic acid), and the colour developed must be compared with the standard within 5 minutes of completing the addition of the reagents to the test solution. It is advisable to maintain the acidity at 2 cc. of 5·5 *N* sulphuric acid per 15 cc. of reaction mixture.

In the present case it is difficult to suggest the actual combining proportions of the molybdate and the pyrophosphate, because of the lack of accuracy in determining the end-point. For example, from Table II, column 5, tube 13, it is found that 85 mg. of sodium pyrophosphate combine with 100 mg. of ammonium molybdate, from which, by calculation, 0·3363 mols. of pyrophosphoric acid combine with 1 mol. of MoO_3. Compounds of pyrophosphates and tungstic acid have been described by Gibbs [1885] of the general formula

$$9M_4P_2O_7,\ 22WO_3,\ xH_2O.$$

From the above calculation the following relation holds:

$$0{\cdot}3363H_4P_2O_7 : MoO_3 :: 7{\cdot}4H_4P_2O_7 : 22MoO_3.$$

In respect, however, to the formulae of complex compounds of these types, it should be remarked that the degree of acidity of the reacting solutions has a very marked influence. Wu [1920] has drawn particular attention to this point.

Sodium glycerophosphate.

The effects of increasing quantities of sodium glycerophosphate on the production of blue colour were very similar to those described above with alkali pyrophosphate. Table III shows the interfering and hydrolytic effects in the acid solution. It was difficult to determine the exact point at which colour just disappeared because the colours were visibly increasing in intensity whilst they were being compared.

Table III.

Tube No.	cc. sodium glycerophosphate	Percentage of phosphorus recovered
1	0	100
2	0·5	118
3	0·75	118
4	1·0	118
5	1·1	95
6	1·2	Gradual
7	1·5	decrease
8	1·9	0
9	2·2	No recovery after an interval of 10 mins.

Each tube contained 1 cc. of potassium dihydrogen phosphate solution containing 0·08 mg. of phosphorus; increasing amounts of sodium glycerophosphate solution (12·24 g. of $Na_2C_3H_7O_6P$, $5H_2O$ dissolved in water and made up to 100 cc., which then contained 0·04 mol. of the salt); 2 cc. of ammonium molybdate solution (6·1815 g. dissolved in water and made up to 100 cc., which then contained 0·005 mol. of the salt); 2 cc. of 5·5 *N* sulphuric acid; 0·5 cc. of 1:2:4-aminonaphtholsulphonic acid; and water to 15 cc. Colours were compared after 5 minutes. When allowed to stand for 12 hours, the contents of all the tubes became deeply coloured.

On the assumption that at the point at which the colour first disappears there are present exactly equivalent amounts of ammonium molybdate and sodium glycerophosphate to form a complex salt, it is possible to suggest the combining proportions of the two substances. By experiment, 1·9 cc. of sodium glycerophosphate solution combine with 2 cc. of ammonium molybdate solution from which, by calculation, 1·08 mols. of glycerophosphoric acid combine with 1 mol. of MoO_3. A series of experiments was carried out using a much smaller concentration of acid (0·5 cc. of 5·5 *N* H_2SO_4 per 15 cc. of total mixture) and showed that the combining proportions were not materially affected.

In a recent paper, Fleury [1932] described a physical and a chemical method for determining complex formation between molybdic acid and alkali glycerophosphates in neutral solution. He also isolated crystalline complex salts from neutral solution of the following composition: $M_2C_3H_7O_6P$, $2{\cdot}5MoO_3$, xH_2O.

It is possible to determine accurately 0·08 mg. of phosphorus present as alkali orthophosphate by the colorimetric method in the presence of less than 20 mg. of sodium glycerophosphate per 0·1236 g. of ammonium molybdate, provided the conditions of the estimation are those described for the accurate determination of phosphate in the presence of alkali pyrophosphate.

The effect of certain organic acids.

Table IV gives a list of certain organic acids which, in sufficient quantity, inhibit the formation of the blue colour due to the reduction of phosphomolybdic acid. In these cases hydrolysis with production of increasing quantities of orthophosphoric acid cannot take place. The point at which colour first disappears when adding increasing amounts of the inhibitor is not so obscure as in the previous cases. Moreover, the range over which accurate determinations of orthophosphate in the presence of these substances can be made is greater than the range in the presence of pyrophosphoric or glycerophosphoric acids.

Table IV.

Interfering acid	Maximum amount in mg. of the interfering acid with which 100 % recovery of phosphorus is possible	g.-mol. of interfering acid added when complete absence of colour is first observed	Combining proportions
Citric acid	30	$3{\cdot}6 \times 10^{-4}$	$0{\cdot}5C_6H_8O_7$, MoO_3
Oxalic acid	50	8·0 ,,	$C_2H_2O_4$, MoO_3
Pyruvic acid	50	10·0 ,,	$1{\cdot}5C_3H_4O_3$, MoO_3
Tartaric acid	90	10·4 ,,	$1{\cdot}5C_4H_6O_6$, MoO_3
Malic acid	120	21·0 ,,	$3C_4H_6O_5$, MoO_3
Lactic acid	120	29·0 ,,	$4C_3H_6O_3$, MoO_3
Glycollic acid	250	64·0 ,,	$9C_2H_4O_3$, MoO_3

The corresponding quantity of ammonium molybdate present was $1{\cdot}0 \times 10^{-4}$ mol., whilst there were 0·08 mg. of phosphorus, and 2 cc. of the standard sulphuric acid. Aminonaphtholsulphonic acid was the reducing agent, and the total volume was finally 15 cc. All the solutions of the interfering substances were exactly neutralised with alkali before use.

A change of the reducing agent to quinol makes no alteration in the figures recorded in Table IV for citric acid. Similarly, with a change of the acidity to 0·5 cc. of the standard acid there is little difference in the figures.

In all cases the colours produced became darker on keeping. Even tubes which originally contained a slight excess of interfering acid became pale blue. Probably the organic acid-molybdate complex is reduced on preservation.

Many methods, both chemical and physico-chemical, have been suggested

for determining complex formation with molybdates. The actual isolation of the complexes is usually very difficult, since they are often non-crystallisable syrups. In general, the results of the various methods appear to be rather conflicting. Darmois [1923] gives a bibliography of previous work on the investigation of mixtures of molybdic acid and other substances. Further work in this direction has been carried out by Fleury [1932]. The experiments reported in the present paper furnish a new method for determining complex formation in acid solution between molybdates and other substances, and give the combining proportions of the two substances. An alkali orthophosphate and a reducing agent are used as indicator of complex formation, and, if the latter takes place, of the point at which all the molybdate is converted into the complex compound. A substance which interferes with the colour development in the manner described herein is probably one which combines with the molybdenum compound. When the interfering substance is in insufficient quantity a blue coloration, which disappears with an excess of the substance, develops. In Table IV combining proportions of molybdenum trioxide and organic acids obtained by this method are tabulated.

The following substances even in large amounts do not interfere with the colour development: formic, acetic, succinic, maleic, fumaric, and malonic acids, alcohol, glycerol, glycine, urea, glucose and fructose. We have not experimented with sufficient compounds to make a generalisation concerning the type of substance which would be likely to undergo complex formation with ammonium molybdate. However, in this connection it should be noted that all the substances given in Table IV contain the carboxyl group, and, in addition, all except oxalic acid contain a hydroxyl or a carbonyl group.

The competitive effects of orthophosphate and interfering substances for molybdate.

The effect of increasing the concentration of orthophosphoric acid in the presence of sufficient sodium citrate to prevent colour formation under standard conditions was investigated. Each tube contained 85 mg. of citric acid and increasing amounts of potassium dihydrogen phosphate. Colour was developed in the usual way. The colour intensity increased proportionately but did not at any time indicate theoretical recovery of orthophosphate. Table V gives the result of the experiment.

Table V. *The recovery of orthophosphate in the presence of sodium citrate.*

Tube No.	Orthophosphate added in mg. of phosphorus	Recovery in mg. of phosphorus
1	0·08	0
2	0·16	0·038
3	0·24	0·047
4	0·32	0·110
5	0·40	0·125

The results show the competitive effect of the orthophosphate and of the citrate for the ammonium molybdate. As the proportion of the phosphate to the citrate increases so the amount of phosphomolybdate increases, whilst the amount of the citric acid-molybdic acid complex must decrease. A complete explanation of the results is bound up with the relative stabilities of the two complex compounds. Reference to Table I, last column, shows a similar effect. When the contents of the tubes are kept for 12 hours, an increased amount of orthophosphoric acid is produced by reason of the hydrolysis of the pyrophosphate in acid solution. This is made evident by the appearance of a blue colour in tubes which originally contained sufficient pyrophosphate to inhibit the colour.

Similar competitive effects have been observed between citric acid and malic acid for ammonium molybdate. We are indebted to Dr J. H. Quastel for providing us with the following results.

Molecular rotations ($[M]_{5461} = \frac{10\alpha}{l.c}$, where α = the observed rotation, l = 2 dm. and c = the molar concentration per 1000 cc. of *l*-malic acid, at room temperature) of aqueous mixtures of ammonium molybdate with varying amounts of citric acid and malic acid are given in Table VI. The solution was made up of 10 cc. of 14·2 % ammonium molybdate solution, 1 cc. of glacial acetic acid, and 5 cc. of the mixture of malic and citric acids. The concentrations of malic and citric acids given in Table VI refer to this solution.

Table VI. *Molecular rotations of mixtures of ammonium molybdate, malic and citric acids.*

		Citric acid concentration				
		0	*M*/160	*M*/80	3*M*/160	*M*/40
Malic acid concentration	*M*/160	+ 1096°	1888°	2096°	2400°	2400°
	M/80	1120	1688	1900	2000	2128
	M/40	1190	1550	1770	1894	—
	3*M*/80	1177	1493	1659	—	—
	M/20	1195	1464	—	—	—

From Table VI it is evident that (1) the molecular rotation of the malo-molybdate complex increases with increase of the concentration of the malic acid to a maximum; (2) for a given concentration of malic acid, an increase in the concentration of citric acid in the mixture produces an increased molecular rotation which finally reaches a maximum. This shows the formation of a citro-malo-molybdate complex of higher rotation than the malo-molybdate complex; (3) for a given concentration of citric acid in the mixture, an increase in the concentration of malic acid reduces the molecular rotation.

These results indicate the replacement of malic acid by citric acid and *vice versa* from their complexes with ammonium molybdate. The trend of the reaction is governed by the relative concentrations of the combining substances. There is obviously competition by the two acids for the available valencies of ammonium molybdate.

Modified method of analysis.

In order to estimate orthophosphate in the presence of certain organic acids, Tschopp and Tschopp [1932] recommended that the latter be removed by oxidation with potassium permanganate. We suggest that the easiest method of obviating the difficulty arising from the presence of the interfering substance is to add sufficient ammonium molybdate to combine with the inhibitor and then the standard quantity to react with the orthophosphate. For example, with citric acid (neutralised with alkali) as inhibitor, 30 mg. are the maximum amount which can be allowed for theoretical recovery of orthophosphate, when using 2 cc. of standard ammonium molybdate (0·1236 g. of the salt). If, however, 50–70 mg. of citric acid are known to be present, then theoretical recovery of orthophosphate will be obtained by adding double the quantity of ammonium molybdate. This method requires no extra step in the method of analysis, but assumes a knowledge of the quantity of interfering substance present. The quantities of the inhibitor which interfere are large, and are much greater than those obtained under physiological or even pathological conditions. This means that they can only be present in such large amounts when they have been added during the course of experimental manipulation. Under these circumstances the amount of the interfering substance can be assessed. From the figures given in Table IV, a calculation can then be applied to find the amount of ammonium molybdate required. In spite of this, it is recommended that the amount of inhibiting substance, and consequently that of extra molybdate, should be kept as low as possible.

With those interfering compounds which through hydrolysis yield free orthophosphate in the acid solution, the above modification is of no value. The quantities present must be restricted to the maximum allowed.

Summary.

1. Pyrophosphoric, glycerophosphoric, citric, oxalic, pyruvic, tartaric, malic, lactic and glycollic acids interfere with the quantitative determination of orthophosphate by the colorimetric method.

2. The maximum amounts of the interfering compounds which may be present for theoretical recovery of orthophosphate have been determined.

3. The anomaly is caused by complex formation between ammonium molybdate and the interfering substance. Besides forming complexes with ammonium molybdate, pyrophosphoric and glycerophosphoric acids cause further difficulties because they undergo hydrolysis producing orthophosphoric acid.

4. From a knowledge of the amount of interfering substance which is necessary just to prevent the formation of blue colour (from reduced phosphomolybdic acid), combining proportions of molybdate and interfering substance have been suggested.

5. By increasing the amount of molybdate, within certain limits, it is possible to estimate orthophosphate in the presence of larger amounts of the foreign substance than are given in (2).

One of us (D. R. D.) is indebted to the Medical Research Council for a whole-time assistance grant.

REFERENCES.

Darmois (1923). *J. Phys. Radium*, **4**, 49.
Davies and Davies (1931). *J. Chem. Soc.* 1207.
Denis and Meysenbug (1922). *J. Biol. Chem.* **52**, 1.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Fleury (1932). *Bull. Soc. Chim.* **51**, 657.
Gibbs (1885). *Amer. Chem. J.* **7**, 313.
King (1932). *Biochem. J.* **26**, 292.
Tschopp and Tschopp (1932). *Helv. Chim. Acta*, **15**, 793.
Vásárhelyi (1929). *Mikrochem., Pregl. Festschr.* 329.
Wu (1920). *J. Biol. Chem.* **43**, 189.

CCXLV. CHOLINE-ESTERASE. AN ENZYME PRESENT IN THE BLOOD-SERUM OF THE HORSE.

By EDGAR STEDMAN, ELLEN STEDMAN and LESLIE H. EASSON.

From the Department of Medical Chemistry, University of Edinburgh.

(*Received October 31st, 1932.*)

The question of the hydrolysis of acetylcholine by enzymes has been brought into prominence in connection with the investigations on the mechanism of nervous action which Loewi has carried out during the past decade. In his original paper on this subject Loewi [1921], working with isolated frogs' hearts, showed that stimulation of the vagus caused the formation or liberation in the heart of a substance which itself produces the effect previously considered to be due to the action of the nerve. It was recognised by Loewi [1922] that this so-called vagus-substance was a choline derivative, while Loewi and Navratil [1926, 1] later advanced evidence which indicated that it was an ester of choline, possibly acetylcholine, although Witanowski [1925], working in Loewi's laboratory, had previously stated that it was more stable than the latter substance. Not only did Loewi and Navratil find that the physiological activities of the vagus-substance and acetylcholine were indistinguishable, but they discovered that both substances were destroyed by aqueous extracts of frog's heart. The destructive agent present in such extracts was thermolabile and possessed other properties characteristic of enzymes; it was therefore considered to be an esterase. Following this work, Galehr and Plattner [1927, 1, 2] demonstrated that acetylcholine was similarly destroyed by defibrinated blood from various species. They were nevertheless of the opinion that no enzyme was involved but that the destruction was brought about by a process of adsorption catalysis, a view which was elaborated in subsequent papers by Plattner and his colleagues. It is unnecessary, however, to consider the latter work in detail since Engelhart and Loewi [1930] have now conclusively demonstrated the enzymic nature of the destructive agent; results of a similar nature have, moreover, been obtained by Matthes [1930].

That acetylcholine should be destroyed by esterases is not surprising. These enzymes, which are fairly widely distributed in the animal organism, are known to hydrolyse a variety of esters, and there seemed no reason to suppose that acetylcholine would be exceptionally resistant to their action. It has long been known, for example, that the blood-serum from many species contains an enzyme which attacks tributyrin, and it appeared logical to attribute the hydrolysis of acetylcholine by blood or serum to the action of this enzyme, and

to assume, in fact, that all esterases, and possibly lipases, would produce the same effect. Nevertheless, as pointed out by Stedman and Stedman [1931], the possibility that the destruction of acetylcholine was effected by a specific enzyme remained. No information was available on this point, for, in the work outlined above, pharmacological methods were employed to follow the destruction of the drug. Owing to the extraordinary activity of this substance, such methods can be and, in fact, were employed with minute amounts of material, the experiments of the above-mentioned authors being carried out with a fraction of a milligram of the drug. While these experiments served to demonstrate the destruction of acetylcholine, it was clear that they could give no information regarding the specificity or otherwise of the enzyme involved; much larger amounts of material would be required for this purpose. Stedman and Stedman therefore attempted to solve this problem indirectly by utilising an observation, made by Loewi and Navratil [1926, 2], that physostigmine, which potentiates the action of the vagus, does so in virtue of the fact that it inhibits the activity of the enzyme which causes the destruction of both acetylcholine and the vagus-substance. Assuming that physostigmine acted specifically on the latter enzyme, as seemed to be indicated by Matthes's results [1930], it appeared that the problem could be readily solved by examining the action of the alkaloid on an authentic esterase. Stedman and Stedman therefore examined the action on liver-esterase, using both methyl butyrate and tributyrin as substrates, of a group of urethanes which they had previously prepared and which, like physostigmine, could be classed as parasympathetic stimulants. In every case the activity of the esterase was inhibited by minute concentrations of the urethanes, a result which afforded strong evidence for the view that the enzyme which destroys acetylcholine is an esterase such as is present in the liver. This view, moreover, received additional support from the subsequent work by the same authors [1932], in which it was shown that whereas the activity of kidney-phosphatase towards glycerophosphoric acid was uninfluenced by the urethanes in question, that of serum-esterase from the guinea-pig towards tributyrin was inhibited to much the same extent as liver-esterase. Further, pancreatic lipase from the pig, although inhibited by relatively large concentrations of the urethanes when methyl butyrate was the substrate, was unaffected when the substrate was olive oil. These results certainly demonstrated that the inhibitory action of urethanes of the type concerned was specific to the extent that it was directed towards true esterases. Indirectly, they seemed to show that the destruction of acetylcholine by blood-serum and tissue extracts was caused by an esterase similar to that present in the liver and not by one of a specific nature.

Action of esterases and lipases towards acetylcholine.

Despite these results it appeared desirable to extend the work and to examine the activity of the various enzymes towards acetylcholine, using chemical methods of estimation. For the first experiments of this kind we

again selected liver-esterase, since preparations of this enzyme containing relatively little protein can be readily obtained by the methods developed by Willstätter and his co-workers [for details, see Stedman and Stedman, 1931]. When, however, the activity towards acetylcholine of such preparations was examined by the procedure described below, none was found. Even with a concentration of enzyme five times as great as that necessary to follow the hydrolysis of methyl butyrate under otherwise identical conditions (p_H 7·5), no hydrolysis of the acetylcholine beyond that due to the hydroxyl ions could be detected. The liver-esterases from two species, namely the pig and the cat, were tested with identical results. Pancreatic lipase from the pig [for preparation, see Stedman and Stedman, 1932] was similarly found to be inactive.

Method of estimation.

In view of these negative results we next turned our attention to the esterase present in horse-serum, this particular species being chosen partly because it had been employed in some of the pharmacological experiments mentioned above and partly because the serum could be readily obtained in quantity. For the estimation, the method of continuous titration, developed by Willstätter, Kuhn, Lind and Memmen [1927] in connection with liver-esterase, and subsequently used by many workers, was utilised under the following conditions. To 100 cc. of water, warmed to 30°, were added a measured volume of a solution of acetylcholine chloride of known concentration, 5 drops of a solution of bromothymol blue, and sufficient 0·0225 N alkali to bring the p_H to about 7·4. A measured volume, usually 1 cc., of the serum or solution to be tested was then added, the flask placed in the thermostat, and, after again adjusting the p_H of the solution, 0·0225 N sodium hydroxide was run into the flask, drop by drop, as required to maintain the p_H of the solution at the above value. Burette readings were recorded every 5 mins. for a period of 20 mins.

While the above method served to demonstrate the hydrolysis of acetylcholine by horse-serum, the results obtained were not as regular as was desired. Two factors were mainly responsible for this: the buffering action of the serum-proteins and the yellow pigment present in the serum. The latter modified the colour of the indicator to such an extent that it became difficult, if not impossible, to maintain the solution at a definite p_H. It was obvious that some purification of the enzyme would have to be effected if its properties were to be examined, and it thus became desirable to devise an improved method of estimation in order to facilitate the process of purification. Two modifications were therefore made in the above procedure. The first depended upon an observation which we made that butyrylcholine is hydrolysed more rapidly by the serum-enzyme than is acetylcholine. The former ester was therefore employed as substrate, a change which possessed a twofold advantage. Not only was there, for a given amount of enzyme, an increased liberation of

acid, but the correction which it was necessary to apply was smaller owing to the fact that butyrylcholine is much less susceptible than acetylcholine to the hydrolytic action of hydroxyl ions. The latter factor was not, it is true, of great importance when the experiments were carried out at p_H 7·4, for at this acidity the correction is almost negligible with either substrate; its advantage lay in the fact that it rendered possible the second modification, which consisted in carrying out the experiments at p_H not less than 8·6, using *o*-cresolphthalein as indicator. At this acidity the buffering action of the serum-proteins was much diminished, and hence the titrations were considerably sharper. Nevertheless, it cannot be claimed that accurate results were obtained with this modified procedure; it was used because it appeared to be the best available for the purpose of tracing the course of purification of the enzyme. When some success had been achieved in this direction, it was possible to revert to the method first described. It should be mentioned that, in order to avoid the possible inhibitory action of alcohol, aqueous solutions of the sodium salts of the indicators, prepared according to Clark [1925], were used throughout the work.

Choline-esterase activity of horse-serum.

It has been shown above that whereas liver-esterase and pancreatic lipase are without action on acetylcholine, an enzyme which is present in the blood-serum from the horse is able to hydrolyse both this substance and butyrylcholine. Although all the solutions of this enzyme which we have hitherto prepared have also attacked both methyl butyrate and tributyrin, their activities towards the latter substrates have been smaller than towards esters of choline. It is possible that different enzymes are involved. Assuming, however, that this is not the case and that the same enzyme has been responsible for the hydrolysis of these different types of esters, it is clear that the enzyme in horse-serum differs from liver-esterase in a manner analogous to that in which the latter differs from pancreatic lipase. It has been shown, for example, by Willstätter and Memmen [1924] that liver-esterase and pancreatic lipase resemble one another in hydrolysing both simple esters and fats, but differ enormously in the relative rates at which they attack these different types of esters, liver-esterase showing a decided preference for the former and pancreatic lipase for the latter type. Similarly, the horse-serum-enzyme, on the above assumption, resembles liver-esterase in hydrolysing methyl butyrate and tributyrin but differs from it in the fact that it also attacks esters of choline, and does so, moreover, more rapidly than it hydrolyses methyl butyrate. In order to emphasise this difference it is proposed to term the enzyme which hydrolyses esters of choline choline-esterase.

The choline-esterase activity of the serum from individual horses varies considerably. This is illustrated by the following experiments. Using, in each case, 0·1 g. of acetylcholine as substrate the acid liberated in 20 mins. at p_H about 7·5 by the enzyme present in 1 cc. of serum was equivalent, with three different sera, to 4·2, 3·6, and 2·45 cc. respectively of 0·0225 N alkali. Two

further specimens were tested at p_H about 8·8 with 0·1 g. of butyrylcholine as substrate; the corresponding figures were 7·2 and 4·65 cc. While, for the reasons explained above, no great accuracy is claimed for these figures, it is evident that wide variations in the choline-esterase content occur.

It is interesting to note that serum, if kept sterile, retains its choline-esterase activity practically unimpaired for months. The procedure we have usually adopted is to saturate the serum with chloroform and then to store it in the refrigerator.

Purification of choline-esterase.

In attempting to purify choline-esterase we have avoided devising a unit of activity for which any permanent meaning could be claimed. We have, however, used two magnitudes in order to give numerical expression to the yield of enzyme obtained and to the degree of purification effected in any operation. These magnitudes may, for convenience, be termed the volume-number and weight-number respectively. The former represents the volume, calculated from a determination of the activity of the preparation, of 0·0225*N* alkali which would be required to neutralise the acid liberated in 20 mins. by the enzyme obtained from 100 cc. of serum; the latter is the volume required to neutralise the acid similarly liberated by that contained in 1 *g.* of the dry material. In calculating the values of these numbers the assumption has been made that the activity is proportional to the enzyme content; this will, of course, only hold strictly under uniform conditions of estimation. During the progress of this work these conditions have been modified; for example, in the earlier experiments only 0·1 g. of acetyl- or butyryl-choline was used as substrate, whereas it was later found advantageous to employ 0·25 g. The values of the volume-numbers and weight-numbers obtained in different series of experiments will not therefore be comparable. Within any single series, however, where the conditions of estimation have been maintained as constant as possible, the values of these expressions give at least an approximate indication of the yield of enzyme which has been obtained and of its degree of purity. Typical experiments, carried out during the attempted purification of the enzyme, are briefly described below.

Dialysis. Collodion membranes were employed throughout these experiments, dialysis being effected under pressure in order to avoid great dilution.

100 cc. of serum were dialysed until salt-free. After removing a precipitate of euglobulin, 130 cc. of solution were obtained. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1·3 cc. required 4·8 cc. alkali in 20 mins. Volume-number, 480; volume-number for original serum, 465. No loss of activity occurs on dialysis.

Precipitation with ammonium sulphate. Preliminary experiments in which attempts were made to remove the enzyme from the dialysed solution by adsorbing it on alumina and kaolin having failed, precipitation with ammonium sulphate was examined. The following experiments give the results obtained with increasing concentrations of the salt.

20 g. of ammonium sulphate were dissolved in 100 cc. of serum. After 2 hours the solution was centrifuged and the centrifugate and precipitate dialysed separately. The precipitate, which readily dissolved in a small volume of water, gave 29 cc. of an almost colourless solution. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 0·85 cc. alkali in 20 mins. Volume-number, 25. The centrifugate yielded 117 cc. of solution containing 3·5 g. of solid. Estimation: 1 cc. required 4·1 cc. alkali in 20 mins. Volume-number, 480; weight-number, 117. Volume and weight numbers for serum, 465, 55.

In another similar experiment the precipitate from 200 cc. of serum was washed with a small volume of ammonium sulphate solution (20 g. salt; 100 cc. water) and the combined centrifugate and washings were dialysed. There were thus obtained 252 cc. of a yellow solution containing 3·6 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 10 cc. of the enzyme solution were diluted to 15 cc., when 2 cc. of this solution required 7·85 cc. alkali in 20 mins. Volume-number, 742; weight-number, 164. Corresponding numbers for serum, 830, 99.

The above results are not considered to be sufficiently accurate to warrant applying corrections for the hydrolysis of the substrate by hydroxyl ions. It is nevertheless clear that the protein precipitated by 20 g. of ammonium sulphate per 100 cc. of serum contains little if any enzyme. By removing this protein a considerable purification is therefore effected. Calculated on the dry-weight, the activity is approximately doubled.

105 g. of ammonium sulphate were dissolved in 300 cc. of serum and, after standing for some hours, the solution was centrifuged. The liquid was rejected. The precipitate was dissolved in water and the solution dialysed. Some protein which separated during dialysis was removed in the centrifuge, when 168 cc. of solution were obtained. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 7·1 cc. alkali in 20 mins. Volume-number, 398 (serum, 465). A large proportion of the enzyme is thus precipitated under these conditions. In view of the fact that a slight loss of material occurred in this experiment owing to a leak in one of the collodion membranes, the following experiment was carried out.

The precipitate obtained by dissolving 40 g. of ammonium sulphate in 200 cc. of serum was removed and washed as described above. The ammonium sulphate content of the combined centrifugate and washings was increased to 35 g. per 100 cc., the precipitate removed in the centrifuge, dissolved in water and dialysed. 83 cc. of solution were obtained containing 11 g. of solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 9·5 cc. alkali in 20 mins. Volume-number, 369; weight-number, 67. Corresponding numbers for serum, 465, 55. It is clear that some enzyme escaped precipitation and that little purification is effected under these conditions.

A further 200 cc. of serum were treated with 40 g. of ammonium sulphate

and the precipitate was washed as described above. The ammonium sulphate content of the combined centrifugate and washings was increased to 38 g. per 100 cc. and sufficient acetic acid was added to render the solution slightly acid to litmus. The precipitate was removed, dissolved in water, and the solution dialysed. There were thus obtained 80 cc. of solution containing 8·4 g. of solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 9·7 cc. alkali in 20 mins. Volume-number, 388; weight-number, 116. Corresponding numbers for serum, 465, 55.

40 g. of ammonium sulphate were dissolved in 100 cc. of serum, the precipitate dissolved in water and the solution so obtained dialysed. The salt-free solution measured 65 cc. and contained 8 g. of solid. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 7·2 cc. alkali in 20 mins. Volume-number, 468; weight-number, 58. Corresponding numbers for serum, 465, 55.

The above experiments demonstrate that the choline-esterase is precipitated with the more soluble proteins. Beyond the purification effected by the removal of the virtually inactive precipitate obtained with 20 g. of ammonium sulphate per 100 cc. of serum, simple fractional precipitation with this salt is evidently useless as a method for increasing the purity of the enzyme.

Glycerol extraction. The 65 cc. of solution obtained in the last experiment of the preceding section were evaporated to dryness at a temperature not exceeding 30°. The solid residue (8 g.) obtained was ground to a fine powder and extracted for 1½ hours at 30° with 90 % glycerol. Undissolved solid was removed in the centrifuge and the solution poured into a collodion membrane and dialysed. The residue was dissolved in water and similarly dialysed. The solution obtained from the extract measured 27 cc. and contained 0·11 g. of solid per cc. Estimation: p_H, 8·8; substrate, 0·1 g. butyrylcholine chloride; 1 cc. required 6·55 cc. alkali in 20 mins. Weight-number, 60; serum weight-number, 55. The residue yielded 24 cc. of solution containing 0·086 g. solid per cc. Estimation: 1 cc. required 5·7 cc. alkali in 20 mins. Weight-number, 66.

Extraction with ammonium sulphate. A quantity of serum was treated with 20 g. of ammonium sulphate per 100 cc., and, after removal of the inactive precipitate, dialysed. The solution so obtained was evaporated to dryness at a low temperature and the residue ground to a fine powder. 2 g. of the latter were extracted with 10 cc. of ammonium sulphate solution (20 g. salt; 100 cc. water). After centrifuging, the solution and residue were dialysed separately. The former gave 20 cc. of solution containing 6·4 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 1 cc. required 9·0 cc. alkali in 20 mins. Weight-number, 140; serum weight-number, 99. The solution obtained from the precipitate measured 9 cc. and had little activity: 1 cc. required 1·35 cc. alkali in 20 mins.

A further 2 g. of the solid were extracted for 1 hour with 10 cc. of ammonium sulphate solution (35 g. salt; 100 cc. water). The mixture was then centrifuged and the solution and residue were dialysed separately. The solution yielded

17·5 cc. of liquid containing 1·9 g. solid per 100 cc. Estimation: p_H, 8·8; substrate, 0·25 g. butyrylcholine chloride; 1 cc. required 5·35 cc. alkali in 20 mins. Weight-number, 282; serum weight-number, 99. The solution from the precipitate measured 19 cc. and contained 7·4 g. solid per 100 cc. Estimation: 1 cc. required 8·0 cc. alkali in 20 mins. Weight-number, 108.

Final method of purification.

The best preparation of choline-esterase which we have hitherto obtained has been prepared by a method based upon the preceding experiments. In this method we have utilised the observations that the protein precipitated with 20 g. of ammonium sulphate per 100 cc. contains little or no enzyme, and that extraction with a solution of 35 g. of ammonium sulphate in 100 cc. of water of the residue obtained by evaporation of the dialysed centrifugate gives a solution in which the ratio of enzyme to protein, as reflected by the weight-number of the preparation, is much increased. Unfortunately, however, the process of evaporation, even when carried out under extremely mild conditions, is accompanied by some destruction of the enzyme. We have therefore attempted to avoid evaporation by the following procedure, which also possesses the advantage that dialysis of large volumes of solution is avoided. Since this procedure has provided us with our best preparation of choline-esterase and has given consistent results with different specimens of serum, a typical experiment is described in detail.

Ammonium sulphate (20 g.) was dissolved in 100 cc. of serum. The precipitate was removed in the centrifuge, washed with a small volume of a solution of ammonium sulphate (20 g. to 100 cc. water) and the mixture again centrifuged. The concentration of ammonium sulphate in the combined centrifugates, which measured 115 cc., was increased to 40 g. per 100 cc. by the addition of 23 g. of the solid salt, and the solution rendered faintly acid to litmus by the addition of 8 cc. of $N/2$ acetic acid. After about an hour, the precipitate which formed was centrifuged off, the liquid being rejected. The precipitate was then stirred in the centrifuge-glasses with about an equal volume of a solution of 35 g. of ammonium sulphate in 100 cc. of water. After standing overnight, the suspension was again centrifuged and the clear liquid poured into a flask. In the course of a few hours a crystalline precipitate, probably of albumin, separated. This was removed, and the concentration of ammonium sulphate in the liquid, which measured 34 cc., was increased to 40 g. per 100 cc. by the addition of 1·7 g. of the solid salt; 0·5 cc. of $N/2$ acetic acid was also added to render the solution faintly acid. A precipitate separated; after a few hours this was removed, dissolved in a small volume of water, and the solution so obtained was dialysed. A small quantity of protein separated during dialysis. This was centrifuged off and rejected, leaving a clear solution with a faint brown colour. Estimation: p_H, 8·8; substrate, butyrylcholine chloride (0·25 g.); 1 cc. of solution, which contained 1·996 % of solid, required 12·85 cc. of alkali in 20 mins. Weight-number, 644; serum weight-number, 99.

When a correction is applied for the hydrolysis of the substrate by hydroxyl ions, these figures become 581 and 82 respectively. This indicates that, based on the dry-weight, the purified preparation was seven times as active as the original serum. The purification actually effected was, however, probably greater than this, for, as previously explained, some uncertainty exists regarding the accuracy of the estimations made with unpurified serum. The exact degree of purification is, however, unimportant. The advantage of the process lies in the fact that the contents of protein and of yellow pigment in the preparation have been reduced to such an extent that the activity of the enzyme can be followed with great accuracy.

Action of choline-esterase on various substrates.

The activity towards various substrates of the preparation of choline-esterase described above was examined at two different acidities, with the results shown in Table I. 1 cc. of the enzyme solution was used in each case.

Table I.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·8	3·25, 3·2, 3·2, 3·2	12·85	1·35	11·5
,,	7·3	3·25, 3·3, 3·0, 3·2	12·75	0·1	12·65
Acetylcholine	8·8	2·2, 2·05, 2·1, 2·05	8·4	2·4	6·0
,,	7·3	1·4, 1·5, 1·35, 1·3	5·55	0·2	5·35
Methyl butyrate	7·3	0·15, 0·2, 0·2, 0·15	0·7	0·1	0·6

As substrates, 0·25 g. of the chlorides of the choline esters and 0·25 cc. of methyl butyrate were employed. The indicators used were *o*-cresolphthalein and bromothymol blue. Corrections for the non-enzymic hydrolysis of the substrates were determined under conditions identical with those used in the enzyme experiments. In addition to these experiments, the action of 1 cc. of the enzyme preparation on tributyrin was examined stalagmometrically under the conditions (p_H, 7·9; T., 20°) described by Stedman and Stedman [1931]. The diminution in the drop number in 40 mins. was 22.

The results with another preparation are given in Table II.

Table II.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·6	1·0, 1·05, 1·1, 1·1	4·35	0·65	3·7
,,	7·4	1·15, 1·15, 1·15, 1·15	4·6	0·1	4·5
Acetylcholine	8·6	1·25, 1·25, 1·1, 1·2	4·8	1·2	3·6
,,	7·4	0·8, 0·85, 0·8, 0·8	3·25	0·15	3·1

In these experiments the enzyme preparation was diluted in order to reduce the rate of hydrolysis of the substrates, which was somewhat too great in the experiments of Table I. Owing to the slower rate at which it was necessary to add the alkali, this permitted a much more accurate control of p_H.

For comparison with the results of Tables I and II, the experiments carried out with 1 cc. of the serum from which the preparations used above were made are recorded in Table III.

Table III.

Substrate	p_H	cc. 0·0225 *N* NaOH used in 5 min. periods	Total	Non-enzymic hydrolysis	Total (corr.)
Butyrylcholine	8·8	2·3, 1·9, 2·1, 2·0	8·3	1·35	6·95
Acetylcholine	8·8	1·7, 1·7, 1·6, 1·45	6·4	2·4	4·0
Methyl butyrate	8·8	0·6, 0·55, 0·45, 0·6	2·2	0·2	2·0

Using tributyrin as substrate with 1 cc. of the same specimen of serum, the diminution in the drop number (p_H, 7·9; T., 20°) in 40 mins. was 23. While great accuracy cannot be claimed for the results in Table III, they are reproduced because they show that the relative activities of the enzyme preparation towards the three types of substrate employed, namely, esters of choline, methyl butyrate and tributyrin, have changed considerably during the process of purification. In particular, the activities towards methyl butyrate and tributyrin are much depressed in the purified preparation. This may indicate that different enzymes are involved, but the data at present available are insufficient to permit of a definite decision on this point.

Summary and discussion.

The preceding experiments have demonstrated that there is present in the blood-serum from the horse an enzyme, for which the name choline-esterase is suggested, which hydrolyses both acetyl- and butyryl-choline, the latter substrate being attacked more rapidly than the former. This enzyme is clearly different from the liver-esterases from the pig and the cat, since the latter are without action on esters of choline. Methods for the purification of choline-esterase are described.

The question of the distribution of the new enzyme has not yet been examined. It must not, however, be concluded from the above results that it is necessarily absent from the liver. The liver-esterase which we have employed has been obtained by extracting desiccated liver powder with dilute ammonia, desiccation of the liver having been effected by the use of acetone and ether. It is probable that such treatment would destroy choline-esterase. Thus, in some preliminary experiments not recorded above an attempt was made to purify the enzyme in horse-serum by precipitating it together with the proteins with acetone and then drying the precipitate by successive treatment with this solvent and ether. The dry preparation so obtained dissolved completely in water, but the resulting solution was without action on acetylcholine.

The presence in horse-serum of an enzyme which hydrolyses tributyrin has long been known. As far as we are aware, however, its action towards simple esters such as methyl butyrate has not hitherto been investigated. The above results now show that this ester, in addition to tributyrin and esters of

choline, is hydrolysed by an enzyme present in the serum. Whether one enzyme is responsible for the hydrolysis of all three types of substrate, or whether different enzymes are involved, cannot yet be decided. That the relative activities of purified preparations of the enzyme towards the three substrates should prove to be different from that of serum must, however, be regarded as evidence in favour of the latter view.

Part of the expense of this investigation has been met by grants from the Earl of Moray Research Fund of this University.

REFERENCES.

Clark (1925). The determination of hydrogen ions. (Baltimore.)
Engelhart and Loewi (1930). *Arch. exp. Path. Pharm.* **150**, 1.
Galehr and Plattner (1927, 1). *Pflüger's Arch.* **218**, 488.
—— —— (1927, 2). *Pflüger's Arch.* **218**, 506.
Loewi (1921). *Pflüger's Arch.* **189**, 230.
—— (1922). *Pflüger's Arch.* **193**, 201.
—— and Navratil (1926, 1). *Pflüger's Arch.* **214**, 678.
—— —— (1926, 2). *Pflüger's Arch.* **214**, 689.
Matthes (1930). *J. Physiol.* **70**, 338.
Stedman and Stedman (1931). *Biochem. J.* **25**, 1147.
—— —— (1932). *Biochem. J.* **26**, 1214.
Willstätter and Memmen (1924). *Z. physiol. Chem.* **138**, 216.
—— Kuhn, Lind and Memmen (1927). *Z. physiol. Chem.* **167**, 303.
Witanowski (1925). *Pflüger's Arch.* **208**, 694.

CCXLVI. VITAMIN C AND THE SUPRARENAL CORTEX.

I. ANTISCORBUTIC ACTIVITY OF OX SUPRARENAL.

By LESLIE JULIUS HARRIS and SURENDRA NATH RAY.

From the Nutritional Laboratory, University of Cambridge and Medical Research Council.

(*Received October 31st, 1932.*)

The chemical study of vitamin C has been notably advanced by the discovery that hexuronic acid possesses antiscorbutic properties. It will be recalled that when he first isolated hexuronic acid, Szent-Györgyi [1928] concluded that it was identical with the reducing substance which is found in active vitamin C concentrates. However, observations by Zilva seemed to show that the reducing substance in question bore no relationship to vitamin C, and hence the matter was dropped for some years. In the meantime a series of detailed investigations by Tillmans and his collaborators [1930; 1932] led to the conclusion that antiscorbutic activity went strictly parallel with reducing capacity and it was argued that the reducing substance was "the carrier of vitamin C activity." Recently the whole question has been re-opened by Svirbely and Szent-Györgyi [1932, 1, 2], who reported that a specimen of hexuronic acid (derived from ox suprarenals) in doses of 1 mg. per day would protect guinea-pigs from scurvy over a 90-day test period. Independently King and Waugh [1932; see also Waugh and King, 1932] have found that a crystalline preparation of vitamin C which they isolated from orange juice possesses the chemical and physical properties of hexuronic acid. The conclusion which Szent-Györgyi draws from his test that "vitamin C is a single substance identical with hexuronic acid" is disputed by Zilva [1932, 1], although he admits [1932, 2] that specimens of hexuronic acid may contain some vitamin C. Zilva [1932, 3] opposes also the conclusions of Tillmans.

In a previous communication from this laboratory [Harris, Mills and Innes, 1932] confirmation was obtained, by means of striking curative tests and also by the microscopic tooth-structure method, of the conclusion which Svirbely and Szent-Györgyi had reached from a preventive test, that hexuronic acid possesses antiscorbutic potency; and its degree of activity was also determined quantitatively. The question was discussed whether the antiscorbutic action of hexuronic acid was an inherent property of the pure substance itself

or whether possibly it was due to traces of some associated impurity. It was pointed out that the theory that the vitamin and hexuronic acid are one and the same substance demands that the raw suprarenal cortex should possess an exceptionally high antiscorbutic activity, since this organ is known to be an even richer source of hexuronic acid than is orange juice itself. This we have found to be the case, the degree of activity of the cortex being in fact proportional to its high content of hexuronic acid[1]. A description of these experiments forms the subject of the present paper[2].

Experimental.

Supplies of fresh ox suprarenal were obtained daily or every second day from local slaughter-houses and used with as little delay as possible, being kept in the mean time in cold store in an atmosphere of CO_2. On removal from cold store, the medulla was dissected away from the cortex, and the latter, after being passed through a fine wire sieve, was suspended in water and immediately administered by means of a dropper to the guinea-pigs used as experimental animals. One object of feeding the cortex only and removing the medulla was to avoid any possibility of adrenaline poisoning. It was also desirable to administer the vitamin in as concentrated a form as possible (there is no evidence of any being present in the medulla) since earlier experience in this laboratory has shown that guinea-pigs fail to thrive if given too large amounts of animal tissue.

The antiscorbutic activity of the suprarenal cortex was determined (1) by curative tests, and (2) by the microscopic tooth structure method.

1. *Curative tests.*

This method has been used successfully in previous work published from this laboratory [*e.g.* Harris, Mills and Innes, 1932; Mills, 1932]. Matched guinea-pigs, males only, weighing about 250 g. are first placed for a preliminary period of about 10 days on the scorbutic basal diet:

Bran	80	parts by weight
Oats	720	,,
Egg-yolk	40	,,
Salts	8·4	,,
Cod-liver oil	1	%

supplemented by 15 g. per day of cabbage, and next on the basal diet alone. Within a further period of 2 to 3 weeks animals are chosen for test which are showing early symptoms of scurvy, have begun to lose weight evenly and have dropped sharply 10 to 20 g. from their maximum weights (in the course of about 3 days) (see figure). Any animals whose weight curves have been lacking in smoothness or who have shown a departure from the average normal response are discarded, as also are those giving indications of scurvy complicated by infection (a not infrequent occurrence).

[1] Preliminary report [Harris, Mills and Innes, 1932].

[2] As reported in an earlier note [Dann *et al.* 1932] no confirmation could be found for the claim of Rygh and co-workers [Rygh and Rygh, 1932, 1; Rygh, Rygh and Laland, 1932] to have identified vitamin C with methyl*nor*narcotine or irradiated narcotine, and the criticisms there made of their technique and their deductions do not seem to be met by their later communication [Rygh and Rygh, 1932, 2].

Graded daily doses of the antiscorbutic supplement are then fed, and the recovery rates noted. A series of graded doses of orange juice (standard) is fed at the same time to another set of animals[1]. Negative controls are also taken and permitted to stay on the scorbutic diet, to

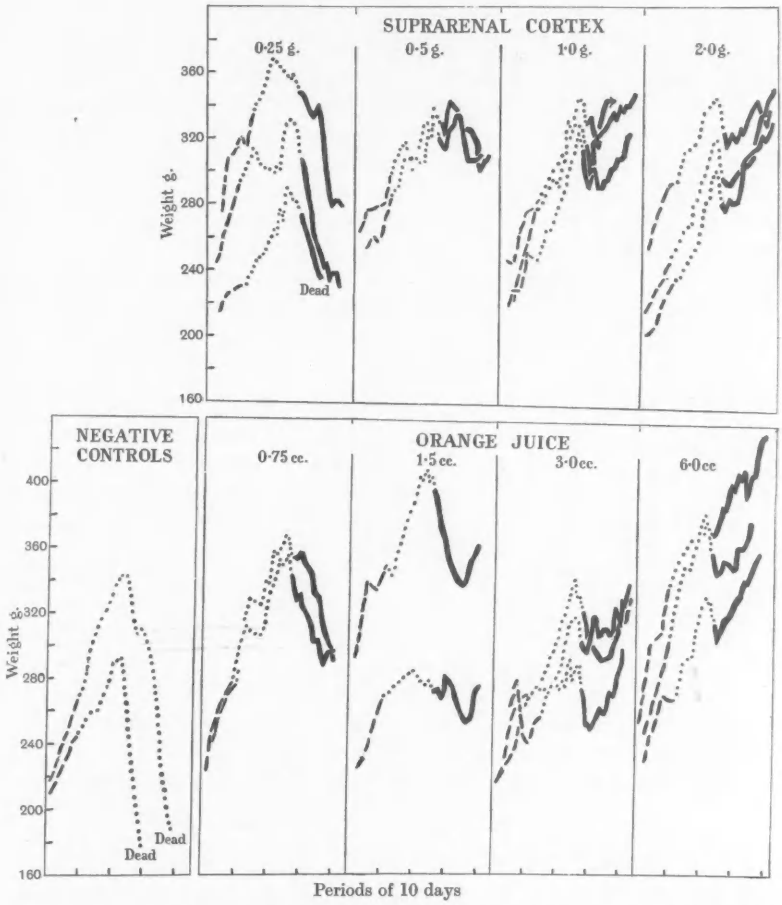


Fig. 1. Weight curves of guinea-pigs, comparing degrees of cure of scurvy with graded doses of suprarenal cortex (above) and orange juice (below).

— — — Preliminary complete synthetic diet.
..... Vitamin C-free diet. ——— Experimental period.

demonstrate that in a given batch or litter the animals are behaving regularly, and would, in fact, continue to lose weight rapidly and die from scurvy but for the addition of the antiscorbutic supplement. After a day or two, growth is resumed at full rate, provided adequacy of vitamin C has been allowed. With partial adequacy subnormal growth results (see Fig. 1), or the decline in

[1] Like Key and Elphick [1931], we have found orange juice preferable as standard of reference to lemon juice (the International Standard).

weight is merely stayed. Thus one can compare the doses of unknown and of standard needed to cause equal resumption in growth rate.

This method possesses a number of advantages. The demonstration of antiscorbutic activity is dramatic and unequivocal. The method is also sparing of material and is of course more rapid than the usual preventive method, since, assuming a continual supply of guinea-pigs developing scurvy, a test can safely be concluded within as short a period as 10 days. Practically the only precaution is to make no use of animals which have declined too far, and would therefore fail to respond. Animals which develop intercurrent infection or other complication to the scurvy, which would likewise prevent a normal response, should be omitted from consideration.

In the present determinations, the suprarenal cortex was tested at four levels, 0·25, 0·5, 1·0 and 2 g., and the results compared with those given by orange juice fed at the four levels of 0·75, 1·5, 3 and 6 cc. Three animals were included in each of these eight groups except that at the two lower levels of orange juice and one on suprarenal cortex only two could be spared. The individual growth curves are shown in Fig. 1. Fig. 2 gives the average gains in weight plotted against the amounts of antiscorbutic material fed.

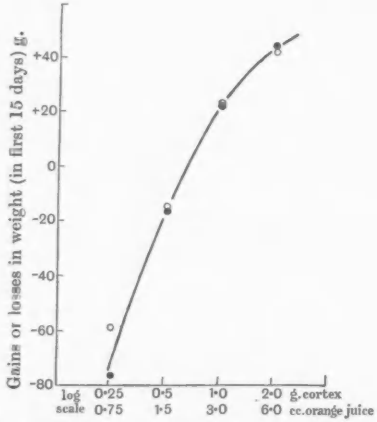


Fig. 2. Vitamin C dosage-response curve, showing average weight gain or loss with given doses of supplements.

○ Orange juice. ● Suprarenal cortex.

Result. It will be seen that 2·0 g. of raw suprarenal cortex corresponded very accurately with 6 cc. of orange juice, 1 g. with 3 cc., and 0·5 g. with 1·5 cc.

2. *Tooth-structure method.*

This method, based on Höjer's work, and depending on preventive action on microscopic tooth structure, has been used with satisfaction in this laboratory for the past 4 years. Normal young guinea-pigs weighing about 300 g. are given the scorbutic basal diet cited above supplemented with suitable graded daily doses of the substance to be tested. Reference animals receive graded doses of orange juice. After 14 days all the animals are killed, and cross sections of the roots of the incisors are prepared by the usual method. Comparison is made between the appearance of the teeth in the groups receiving unknown and standard (orange juice); preferably the amounts may be compared which just suffice to permit the development of normal tooth structure.

In a preliminary experiment raw suprarenal cortex was tested at levels of 0·5, 1·0 and 2·0 g., using three animals at each level. For reference orange juice was fed at levels of 1·5, 3 and 6 cc. Negative controls were given the basal diet alone.

The result of this test (Table I) showed that the minimum dose of suprarenal cortex needed for complete dental protection was approximately 1·0 g., compared with 3 cc. for orange juice.

Table I.

Material tested	Daily dose	Degree of protection on Key and Elphick's [1931] scale ("4" represents full protection)
Suprarenal cortex	0·5 g.	2, 2, 1 (av. 2)
,,	1·0 g.	4, 4, 4 (av. 4)
,,	2·0 g.	4, 4, 4 (av. 4)
Orange juice	1·5 cc.	3, 2 (av. 2–3)
,,	3·0 cc.	4, 4 (av. 4)
,,	5·0 cc.	4, 4 (av. 4)
Negative control	(No supplement)	0, 0 (av. 0)

In a confirmatory test 5 guinea-pigs were each given 0·75 g. of suprarenal cortex daily, an amount judged to be sufficient to produce a moderate degree of protection. Reference animals had 2 cc. of orange juice. The results (Table II) are in keeping with the two earlier assays.

Table II.

Material tested	Tooth sections, degree of protection in individual animals
Suprarenal cortex, 0·75 g. daily	3, 2, 2–3, 1, 2 (av. 2–3)
Orange juice, 2·0 cc. daily	2–3, 2 (av. 2–3)
Negative control (basal diet only)	0–1, 0–1 (av. 0–1)

Experiments with guinea-pig suprarenals.

Preliminary experiments on normal guinea-pig suprarenals show them to be comparable with ox suprarenals in vitamin C activity. Suprarenals from scorbutic guinea-pigs on the other hand were devoid of vitamin C (see Table III). Fuller details will be published in a later paper.

Table III.

Diet	Weight gains or losses during first week of curative test (g.)
Basal diet alone (negative control)	−72, −39
Suprarenal glands (whole) from scorbutic guinea-pigs, 0·5 g. per day	−81, −82
,, ,, normal ,, ,,	+15, −39

Discussion.

1. Vitamin C and hexuronic acid.

The first conclusion emerging from our results is that the relative antiscorbutic activities of suprarenal cortex and orange juice are exactly proportional to the amounts of hexuronic acid which can be isolated from these

two sources. According to Szent-Györgyi [1928], three times as much hexuronic acid can be recovered from a given weight of suprarenal cortex as from orange juice (300 mg. and 100 mg. per kg., respectively), and our experiments show that raw suprarenal cortex possesses also precisely three times the antiscorbutic activity of orange juice. This result is therefore in excellent conformity with the theory (although of course not conclusive evidence for it) that vitamin C is identical with hexuronic acid, assuming the same degree of recovery of hexuronic acid from the two sources.

Actually it is easy to show that a considerable loss of antiscorbutic material occurs during the course of isolation of the hexuronic acid in both cases. Thus, from 1 kg. of suprarenal cortex 300 mg. of hexuronic acid were isolated [Szent-Györgyi, 1928], representing according to the results of Harris, Mills and Innes not more than 300 minimum protective doses (Höjer), as compared with about 1000 doses in the original kg. of cortex itself. The loss is therefore fully two-thirds. The percentage loss is presumably identical with that from orange juice, the hexuronic acid recovered from the two sources possessing the same antiscorbutic activity (see below). While admittedly, the evidence for identifying vitamin C with hexuronic acid would have been more conclusive had the whole of the antiscorbutic activity of the original raw material been successfully concentrated in the form of the hexuronic acid separated from it, it is nevertheless too much to expect that no loss should occur when working with so unstable a material as vitamin C; and the fact that the percentage loss is the same when the same process is applied to widely different raw materials might be regarded as evidence for rather than against the theory. Furthermore the loss of vitamin seems to run parallel with that of hexuronic acid, since Szent-Györgyi [1927; 1928] estimates the amount of the latter actually present in the cortex to be two or three times that which can be recovered from it.

It is also possible to account approximately for the antiscorbutic activity of the cortex, or of orange juice, on the basis of its total hexuronic acid content, estimated chemically. For cortex, this is from 600 to 1000 mg. per kg. according to Szent-Györgyi's [1927; 1928] data; say 1000 mg. Now the minimum (Höjer) dose of hexuronic acid is from 1 to 2 mg. according to the findings of Harris, Mills and Innes; say 1 mg. Hence there are 1000 doses of 1 mg. each in the 1000 mg. of hexuronic acid in the 1 kg. of cortex. That is, the minimum dose of cortex is 1 g. This agrees with our experimental finding. But it is only by thus choosing maximum values both for the hexuronic acid content and for the activity of the hexuronic acid, that values in such good accordance with observation are reached. (This applies also in calculating the activity of other sources from their hexuronic acid contents.) The choice of the maximum value for the activity of hexuronic acid may be justifiable since it is possible that the observed value is low on account of the difficulty of preventing some inactivation. Again it seems probable that the data for hexuronic acid contents as originally given by Szent-Györgyi were under-

estimated. Thus, in good conformity with the hexuronic acid theory, we have found that the titration values of hexuronic acid and of orange juice as determined under suitable conditions by means of the reduction indicator, 2:6-dichlorophenolindophenol [Tillmans, 1930], are proportional to their relative antiscorbutic activities.

Vitamin history, however, shows the danger of assuming without the most compelling evidence that the activity of an apparently pure material is necessarily due to the main constituent and not to some unsuspected impurity. Now if one supposes the pure vitamin C to have a minimum dosage at all comparable with that of the other vitamins (D, A or B_1), it follows that the activity of hexuronic acid must be due to traces of some associated impurity, amounting to no more than a small fraction of 1 %, the presence of which could easily be overlooked. It will be admitted however that there is no *a priori* justification for arguing the dosage from analogy with the other vitamins. And, while giving full weight to the above considerations, the existing evidence seems to contain nothing definitely discordant with the theory that vitamin C is identical with hexuronic acid; while, speaking more positively, it seems hard to escape the conclusion that the vitamin if not actually hexuronic acid itself is at any rate a substance possessing a close similarity to it both in its distribution and in its chemical behaviour. Whatever the final solution to the problem, this undoubtedly represents an important advance.

The evidence for the above conclusion may be summarised briefly as follows. (1) Vitamin C so far as is known has a similar distribution in nature, to hexuronic acid (*viz.* in suprarenal cortex, orange juice, cabbage[1], and (?) lemon juice). (2) The specimen of hexuronic acid from suprarenals examined by us had the same degrec of antiscorbutic activity as those derived from vegetable sources, tested elsewhere. (3) The widely different processes used for the isolation of (*a*) hexuronic acid by Szent-Györgyi and (*b*) vitamin C by King gave a final material possessing identical antiscorbutic activities and, apparently, chemical and physical properties. (4) Vitamin C closely follows hexuronic acid in the various precipitation and solubility reactions used in the isolation processes. (5) None of the known properties of the antiscorbutic factor is inconsistent with it being hexuronic acid. (6) As pointed out by Szent-Györgyi, Tillmans' observations on the association of antiscorbutic activity with reducing capacity are explicable on the hexuronic acid theory. Svirbely and Szent-Györgyi [1932, 2] have offered an explanation of Zilva's apparently contradictory findings in this direction. Zilva's objection [1932, 1], that he has obtained concentrates with greater activity than hexuronic acid, has been replied to by Svirbely and Szent-Györgyi [1932, 2].

[1] Szent-Györgyi [1928] isolated 250 mg. of hexuronic acid from 5 kg. of cabbage. Making an allowance for his acknowledged greater loss with this material the yield is in good agreement with that calculated from the antiscorbutic potency in comparison with orange juice and suprarenal. Lemon juice has the same antiscorbutic activity as orange juice and apparently a similar content of hexuronic acid although it would seem that the precise figure for the latter has not previously been determined.

The evidence of the present paper is of a remarkable degree of parallelism between hexuronic acid and antiscorbutic activity. The question can only be finally settled by further work showing whether this parallelism is invariable and complete.

2. Vitamin C and the physiology of the suprarenals.

Our results show that the suprarenal cortex possesses an astonishingly high antiscorbutic activity, hitherto quite unsuspected. The organ is considerably more potent in vitamin C than any natural source hitherto known, and certainly contains the vitamin in far greater concentration than any other animal tissue so far investigated. The liver only [Mills, 1932] may contain a greater total quantity of vitamin C, by virtue of its large bulk, but it does not compare with the suprarenal so far as localised concentration is concerned.

What is the significance of the unique collection of vitamin C in this site? The possibility suggests itself that as the liver may serve as a storehouse for vitamin C, so the suprarenal may perhaps be concerned in its elaboration or utilisation. On the other hand, it seems likely that the vitamin may be needed by the suprarenal for its functional activities—as in maintaining adrenaline-like substances in a reduced condition. One of us [Harris, 1931] has shown that the dog, unlike the guinea-pig, monkey or man, is able to synthesise its own vitamin C when none is present in the diet, and the same appears to apply to many other species, including the rat [Parsons, 1920]. Tests are in progress to determine the behaviour of the suprarenal and whether it continues to act as a source of vitamin C in such species under these conditions. We have shown that in the guinea-pig, which by contrast is unable to synthesise its own vitamin C, the suprarenal loses its antiscorbutic activity coincidently with the onset of scurvy. The idea that the suprarenal has a specially intimate physiological connection with vitamin C gains support from the observation [McCarrison, 1920] that it undergoes highly characteristic hypertrophic changes in scurvy.

SUMMARY.

Raw suprarenal cortex (ox) is shown to be a more powerful antiscorbutic than any natural source hitherto known, possessing three times the activity of fresh orange juice (or 30 International Units per g.).

The degree of activity corresponds precisely with the value predicted on the basis of the relative yields of hexuronic acid obtainable from the two sources. (There is however a considerable loss of antiscorbutic material (the same in the two cases, *viz.* over 50 %) and of hexuronic acid during the course of the isolation.) The antiscorbutic potency of the cortex can also be accounted for on the basis of its total hexuronic acid content, as estimated chemically, provided certain assumptions are made in the calculation.

From the foregoing and other evidence it is concluded that vitamin C is either identical with hexuronic acid or is a substance possessing a close

similarity to it in its distribution and in its chemical nature, judging by its behaviour towards various solvents, precipitating agents, *etc.*

Preliminary results show that in guinea-pigs—a species which resembles man and monkeys but differs from many others, including dogs and rats, in being unable to synthesise vitamin C *in vivo* when none is provided in the food—the vitamin C activity of the suprarenal is lost with the onset of scurvy. It is supposed that vitamin C plays a special rôle in the physiology of the suprarenal. The occurrence of distinctive hypertrophic changes in the suprarenals in scurvy is in keeping with this.

REFERENCES.

Dann, Forsyth, Harris, Mills and Innes (1932). *Lancet*, ii, 237.
Harris (1931). Quoted by Innes, *Ann. Rep. Inst. Animal Path., Camb.* 1931. p. 150.
—— Mills and Innes (1932). *Lancet*, ii, 235.
Key and Elphick (1931). *Biochem. J.* **25**, 888.
King and Waugh (1932). *Science*, **75**, 357.
McCarrison (1920). *Ind. J. Med. Res.* **1**, 188, 284, 633.
Mills (1932). *Biochem. J.* **26**, 704.
Parsons (1920). *J. Biol. Chem.* **44**, 587.
Rygh and Rygh (1932, 1). *Z. physiol. Chem.* **204**, 114.
—— —— (1932, 2). *Z. physiol. Chem.* **211**, 275.
—— —— and Laland (1932). *Z. physiol. Chem.* **204**, 105.
Svirbely and Szent-Györgyi (1932, 1). *Nature*, **129**, 576, 690.
—— (1932, 2). *Biochem. J.* **26**, 865.
Szent-Györgyi (1927). *Nature*, **119**, 782.
—— (1928). *Biochem. J.* **22**, 1387.
Tillmans (1930). *Z. Untersuch. Lebensm.* **60**, 34.
—— Hirsch and Dick (1932). *Z. Untersuch. Lebensm.* **63**, 267.
—— —— and Hirsch (1932). *Z. Untersuch. Lebensm.* **63**, 1.
—— —— and Jackisch (1932, 1). *Z. Untersuch. Lebensm.* **63**, 241.
—— —— —— (1932, 2). *Z. Untersuch. Lebensm.* **63**, 276.
—— —— and Siebert (1932). *Z. Untersuch. Lebensm.* **63**, 21.
Waugh and King (1932). *J. Biol. Chem.* **97**, 325.
Zilva (1932, 1). *Nature*, **129**, 690.
—— (1932, 2). *Nature*, **129**, 943.
—— (1932, 3). *Biochem. J.* **26**, 1624.

CCXLVII. THE IODIMETRIC TITRATION OF CYSTEINE AND ALLIED SUBSTANCES.

By COLIN CAMERON LUCAS[1] and EARL JUDSON KING.

Department of Medical Research, Banting Institute, University of Toronto.

(*Received November 1st, 1932.*)

The increasing interest of biochemists in sulphydryl compounds has led to the necessity of finding some method for the accurate determination of these substances. That the problem is complex may be judged from the number of papers proposing new methods or modifications of old ones. The original procedure of Rosenheim and Davidsohn [1904] for the volumetric estimation of thiol acids by the use of iodine in the presence of sodium bicarbonate was found by Klason and Carlson [1906] to give too high values. However, in neutral or acid solutions the reaction between sulphydryl compounds and iodine was shown to be quantitative. Nearly all subsequent iodimetric procedures are based on this observation.

The sulphydryl compounds of especial biochemical interest, cysteine, glutathione and ergothioneine, appear to be peculiarly sensitive to the acidity and temperature of the reaction mixture in which they are titrated. It has been known in a general way that cysteine is oxidised to cystine by iodine and to cysteic acid by bromine. The bromate and iodate methods for cysteine described by Okuda [1925] are based on these observations. Okuda showed that on adding standard bromate to an acid solution of cysteine containing excess of bromide, quantitative oxidation to the sulphonic acid occurred. The concentration of the bromide had no effect as long as it was in excess; the concentration of acid affected only the rate, but not the nature of the reaction, and temperature had almost no effect. However, in spite of the simple course of the reaction, the procedure is not suitable for use on protein hydrolysates, since tyrosine, histidine and tryptophan all react with bromine. Since cystine alone of the amino-acids reacts rapidly with iodine in acid solutions, Okuda studied a similar iodate method. The reaction is somewhat complex, a mixture of disulphide and sulphonic acid (and possibly other products) being formed. The temperature and concentration of acid in the mixture have a pronounced effect on the course of the reaction, which is influenced to a less degree by the concentration of iodide [Okuda, 1924] and other substances present. That this is also true of glutathione has been shown by King and Lucas [1931], who found the amount of iodine required by glutathione to be influenced by both temperature and acidity, and by Perlzweig and Delrue [1927], Gavrilescu [1931]

[1] Holder of the Dr James Page Rutherford Research Grant.

and others, who showed that the presence of added potassium iodide lowered the iodine consumption of glutathione solutions. In the hope of gaining some useful information concerning the conditions under which these compounds are most favourably titrated, a study has been made of the influence of acidity, temperature and concentration on the reaction between iodine and cysteine and several allied substances.

Experimental.

The sulphydryl compounds used in this investigation were dissolved in freshly boiled and cooled distilled water, and, unless otherwise stated, diluted to make approximately 0·04 *M* aqueous solutions. 1 cc. of solution was added to 11 cc. of buffer mixture made up with oxygen-free water. The buffers used were (1) Walpole's *M* sodium acetate + HCl, (2) the 0·1 *M* glycine-NaCl + HCl mixture of Sørensen, (3) Walpole's acetic acid + sodium acetate mixture, (4) McIlvaine's Na_2HPO_4 + citric acid, (5) Sørensen's NaH_2PO_4 + Na_2HPO_4 and (6) the sodium veronal buffer of Michaelis. In some of the work the Universal Buffer Mixture sold by British Drug Houses was used. After mixing the buffer and test solution the p_H was always checked by a capillator measurement before the titration was performed. From time to time the p_H was measured after the titration was completed, to make sure that the buffering action had been effective.

If no indicator is used in the iodimetric titration, a point is reached at which the iodine itself suddenly gives a yellow coloration to the solution. Okuda [1923; 1924; 1925] utilised this in his iodate method for cysteine. This end-point is sharp and readily reproducible, but the iodine used is always appreciably in excess of that required by theory if the reaction occurs according to the equation $2XSH + I_2 \rightarrow XSSX + 2HI$. The use of starch as indicator is not only much simpler, according to Uyei [1926], Blanchetière and Melon [1927] and Turner [1929], but appears to give more accurate results for glutathione. It has the disadvantage that in the case of cysteine and its derivatives, the end-point is not sharp—a pale purplish-blue, fading rapidly, precedes the intense blue characteristic of the starch-iodine complex, rendering doubtful the exact end-point. However, with a little experience, it was found possible to determine this point within 0·2 to 0·3 cc. in 10 cc., *i.e.* less than 3 % error. Tunnicliffe [1925] used nitroprusside as an external indicator in applying the iodine titration procedure of Klason and Carlson [1906] to the estimation of glutathione. Later, Kennaway and Hieger [1927] improved the nitroprusside indicator technique. To a series of small test-tubes they add 2 cc. saturated ammonium sulphate, 2 drops strong ammonia and 5 drops of fresh 1 % nitroprusside solution. 5 to 10 drops of the titration mixture are added to the indicator in the test-tubes. Procedures involving the use of outside indicators are not only troublesome but are scarcely suitable for a micro-method. Moreover as the quantity of thiol compound left in solution becomes small, the nitroprusside colour fails to develop proportionately. We

have found that in the case of glutathione, for example, when only 0·01 to 0·02 mg. per cc. is left in the reduced form, no colour at all appears. Hence a correction factor must be added when nitroprusside is used. The authors add to the observed quantity of glutathione 0·015 V mg., where V is the volume of solution at the apparent end-point. Such a correction factor has been noted by several workers, although many appear to have neglected to use it.

Cysteine.

The effects of temperature and p_H were first studied, since these factors exert the most marked influence on the iodine-combining power of cysteine. The potassium iodide factor was kept constant, either at the lowest possible level by using standard iodine instead of iodate, which requires the presence of an excess of iodide, or by adding a definite amount of iodide to the solution and titrating with iodine. Most of the figures were obtained by direct titration, but in some cases an excess of iodine was added to the cysteine solution and the determination completed by a back titration with 0·01 N thiosulphate. Both methods gave comparable results at 0°. The marked temperature effect when the titration was performed in N hydrochloric acid is shown in Fig. 1.

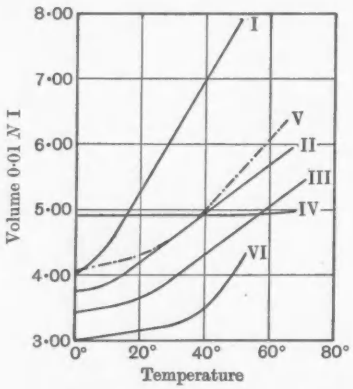


Fig. 1. Effect of temperature on the iodine consumption of sulphydryl compounds: cc. 0·01 N I combined by 1 cc. of (I) 0·04 M cysteine, (II) 0·037 M cysteine ethyl ester, (III) 0·034 M acetylcysteine ethyl ester, (IV) 0·049 M thiophenol, (V) 0·04 M thioglycollic acid, (VI) 0·03 M thiolactic acid.

Influence of acidity on iodimetric titration of cysteine. In order that the temperature of the test solution should not rise above 0°, the vessel was kept in a large dish of brine at about − 5° and the iodine added at a rate not exceeding 1 cc. per minute, the solution being stirred by means of a thermometer. The lowest iodine consumption, compatible with a reasonably fast rate of reaction, took place in solutions N with respect to strong mineral acid (hydrochloric). The reaction under these conditions, and at this dilution (1 cc. of 0·04 M cysteine in 12 cc.) conforms closely to the amount of iodine required

for oxidation to cystine, *i.e.* 4·00 cc.[1] The same quantity of cysteine in an equal volume of 0·1 *N* HCl required from 4·6 to 5·1 cc. of iodine. Although the end-point was sharper in the sense that a blue colour appeared more suddenly the values were quite variable and were not exactly reproducible. Decreasing the acidity by the use of various buffers led to surprising variations in the iodine-combining capacity of cysteine. In spite of careful temperature and p_H control, it was found impossible to get good checks, irregularities of from 0·1 to 0·6 cc. (depending upon the p_H) being apparently unavoidable. The results are best shown graphically (see Fig. 2).

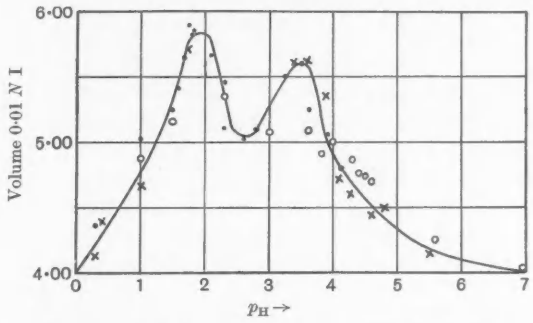


Fig. 2. Effect of p_H on the iodine consumption of cysteine: 1 cc. 0·04 *M* cysteine in a volume of 12 cc. titrated at 0°. (×) HCl and acetic acid-acetate buffers, (o) glycine, citrate and phosphate buffers, (•) B.D.H. Universal Buffer Mixture.

With decreasing acidity increasing amounts of iodine are required, until a maximum iodine consumption is reached at a p_H of about 1·8. The iodine requirement then falls to a minimum at p_H 2·7, rises to a second maximum at p_H 3·5, falls again to a minimum at p_H 7·0, and rises again on the alkaline side of neutrality. At first it was imagined that these peculiar titration values might be in some way related to the buffer solutions, but the same curve was obtained regardless of what buffers were utilised.

In solutions more acid than *N* the starch blue colour appears before the theoretical end-point, fades more or less rapidly, depending on the acidity of the medium, and is not readily determined by direct titration. Titration mixtures were set up as follows:

N: 1·0 cc. concentrated HCl + 1·00 cc. cysteine solution + 10·0 cc. water,
2*N*: 2·0 cc. concentrated HCl + 1·00 cc. cysteine solution + 9·0 cc. water,
3*N*: 3·0 cc. concentrated HCl + 1·00 cc. cysteine solution + 8·0 cc. water.

Because of the end-point difficulty the iodine was added dropwise to 4·0 cc. (the theoretical end-point), the apparent end-point being noted, and then run into 10·0 cc. After the acidity was adjusted (by adding the calculated extra

[1] This agreement may be fortuitous since, in the light of data presented in Table II, it would appear that at other dilutions different amounts of iodine are required.

amount of HCl) the mixtures were set aside at 0° in the dark for the times shown in Table I. From the amount of 0·01 *N* sodium thiosulphate required for back titration, the iodine consumption was calculated. The influence of increasing acidity is seen to be a slight slowing of the first reaction, namely, oxidation to disulphide and a marked inhibition of secondary reactions.

Table I. *Influence of high acidity on uptake of iodine by cysteine.*

HCl	I used at apparent end-point cc.	I used in 5 min. cc.	I used in 20 min. cc.	Increased I uptake in 15 min. cc.
N	4·05	4·20	4·75	0·55
2*N*	3·00	4·28	4·34	0·06
3*N*	2·2	3·91	3·91	0·00

Influence of cysteine concentration on iodimetric titration. The effect of dilution upon the iodine consumption of cysteine solutions has already been investigated by Bierich and Kalle [1928] in connection with their studies on the sulphydryl compounds in tissue extracts. By means of their so-called "dilution factor," which is very large for cysteine, they proposed to distinguish between cysteine and glutathione as iodine absorbers in such solutions. The object in the present investigation was not to devise a method of distinguishing sulphydryl compounds by this means, but to record the magnitude of the errors involved by dilution.

All titrations were performed at 0°, 0·01 *N* iodine being added dropwise to the well-cooled mixture containing 1 cc. of 0·04 *M* cysteine solution in a volume *V* (which initially was *N* with respect to HCl). The theoretical titration was 4·00 cc. The values obtained are contained in Table II.

Table II. *Influence of cysteine concentration on uptake of iodine.*

Constant quantity of cysteine in varying volumes.

Volume cc.	0·01 *N* I used cc.
8	3·9
12	4·0
16	4·4
24	4·8
32	5·0

Table III. *Influence of cysteine concentration on iodine consumption.*

Constant volume, varying amounts of cysteine.

Vol. of cysteine solution in total vol. of 12 cc., cc.	Volume 0·01 *N* I Required cc.	Theory cc.	Difference cc.
1·00	2·7	2·3	0·4
2·00	4·9	4·6	0·3
3·00	7·2	6·9	0·3
4·00	9·3	9·2	0·1

A somewhat similar series with the volume constant and varying cysteine concentrations gave similar results as shown in Table III. Varying volumes of

a cysteine solution containing 0·023 milli-equivalents per cc. were made up to a final volume of 12·0 cc., so that the acidity with respect to HCl was *N*. It is seen that the stronger the cysteine solution, the less is the extent to which iodine in excess of the theoretical is consumed.

The influence of iodine concentration. All titrations were performed at 0° on solutions *N* with respect to HCl, and containing 1 cc. 0·04 *M* cysteine solution in an initial volume of 12 cc. Iodine of different strengths was added dropwise to the well-cooled solutions. The theoretical titration was 4·00 cc. 0·01 *N* I. The values determined are contained in Table IV, where it is seen that the departure from the theoretical consumption is greater when standard iodine solutions of low normality are used.

Table IV. *Influence of iodine concentration on titration of cysteine.*

Normality of I used	Volume I		Difference		
	Required cc.	Theory cc.	cc.	%	As 0·01 *N* I cc.
0·010	4·03	4·00	—	—	—
0·008	5·50	5·00	0·50	10	0·4
0·006	7·80	6·66	1·14	17	0·6
0·004	11·90	10·00	1·90	19	0·8

From a consideration of Tables II, III and IV, it is evident that dilution of either the cysteine solution or the iodine introduces errors which increase with the dilution, but not in any exact ratio to it. In stronger solutions oxidation to the disulphide only occurs, but at the dilutions at which protein hydrolysates and tissue extracts are titrated, the side-reactions introduce a considerable error.

Order of addition of reagents. The influence of the order of addition of reagents was studied briefly. Iodine was added in excess to cysteine solutions and in other cases cysteine was added to excess iodine solution, but although the amounts of iodine combined at different hydrogen ion concentrations were altered, in neither case were the results much closer to the stoichiometric relationship than by direct titration. The result of adding a considerable excess of iodine to the cysteine solution at 0° and various acidities followed by back titration with thiosulphate is shown in Fig. 3. The results in the acid range are uniformly lower than those obtained by direct titration. The first maximum of iodine consumption (at p_H 1·8) is completely obliterated, whereas the second maximum (at p_H 3·5) remains. Iodine consumption falls to a minimum at p_H 6 and rises fairly sharply with increasing p_H, much more iodine being combined at p_H 7 than is the case with direct titration.

Influence of potassium iodide on iodimetric titration of cysteine. Okuda's [1925] observation, that in his iodate method the presence of an excess of potassium iodide lowered the iodine consumption of cysteine solutions, led to an investigation of its influence in the hope that side-reactions might be entirely inhibited. Because of the premature development of the blue colour

in the presence of added iodide, it was found expedient to add an excess of iodine to the cysteine solution and to titrate the excess with thiosulphate. The marked effect of added iodide in lowering the iodine consumption, particularly at acidities less than N, is shown in Fig. 3: 0·5 % potassium iodide partially eliminates the second maximum of iodine consumption at p_H 3·7, and 2 % iodide almost completely.

It is perhaps worthy of note that the shape of the curve relating iodine consumption to p_H for cysteine differs markedly from that for a trichloroacetic acid extract of liver, the iodine consumption of which is constant between p_H 0 and 5, as was shown by King, Baumgartner and Page [1930]. The curve for liver extracts is similar in shape to that for glutathione, and the absence of any

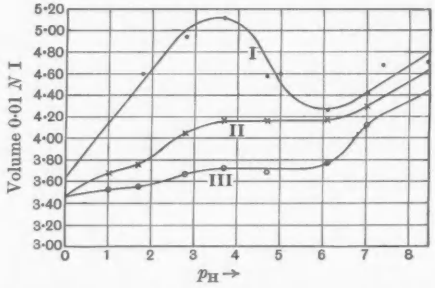


Fig. 3. Influence of p_H on the indirect titration of cysteine: excess iodine added to 12 cc. buffered cysteine hydrochloride solution at 0°, containing (I) no added KI, (II) 0·5 % added KI, and (III) 2 % KI. Excess iodine titrated with 0·01 N sodium thiosulphate.

peaks from the curve, similar to those which are so characteristic of cysteine, would appear to indicate the absence of appreciable amounts of free cysteine from liver.

Secondary oxidation products. The nature of the products formed by the iodine used in excess over that required for oxidation to disulphide, has not been investigated fully, but by allowing a series of tubes containing cysteine to stand for some days with twelve times the iodine necessary for oxidation to disulphide (or twice that necessary for oxidation to cysteic acid) and titrating with thiosulphate at intervals, evidence of eventual oxidation to cysteic acid was obtained. It was found that after 50 hours at room temperature the iodine consumed by cysteine was 92 % of that required by theory for oxidation to cysteic acid. Data from a similar experiment with cystine, as shown in Table V, demonstrated that the oxidation to cysteic acid proceeded to the same point in 50 hours and to 94 % of completion in 100 hours. Since this work has been completed, a paper has appeared by Shinohara [1932] in which the isolation of cysteic acid from the oxidation products of cystine by iodine has been described.

The data set forth in the preceding sections make it plain that no simple rules can be laid down which will ensure quantitative results for the estimation

Table V. *Rate of oxidation of cystine by excess of iodine.*

4·8 mg. cystine in 5 cc. water + 5·0 cc. 0·10 *N* I and 1 cc. conc. HCl, temperature 20°.

Time hours	0·01 *N* $Na_2S_2O_3$ cc.	0·01 *N* I used cc.
0	51·2	0·00
1	47·0	4·2
2	45·7	5·5
4	43·2	8·0
8	39·0	12·2
24	33·2	18·0
50	32·9	18·3
101	32·4	18·8
		Theory 20·0

of cysteine in solutions of unknown strength. Temperature, acidity, concentration (both of the cysteine and of the iodine), the rate and order of addition of reagents and the presence of potassium iodide in varying amounts all affect the iodine consumption in variable ways and to different degrees. Probably the conditions which give results most uniformly according with theory over a wide range of concentration, are indirect titration at 0°, *N* acidity with respect to strong acid and the presence of 0·5 % potassium iodide. Table VI contains the results of an experiment in which cysteine at different dilutions was titrated under these conditions. The results are probably as good as can be obtained under any single set of conditions for the iodimetric titration of cysteine.

Table VI. *Titration of cysteine in different dilutions at 0°, N acidity and 0·5 % KI concentration.*

1 cc. *M*/12 cysteine hydrochloride in different volumes.

Volume cc.	0·1 *N* I cc.	0·1 *N* $Na_2S_2O_3$ cc.	0·1 *N* I combined cc.
1	2·00	1·200	0·800
2	2·00	1·200	0·800
5	2·00	1·200	0·800
10	2·00	1·210	0·790
20	2·00	1·200	0·800
50	2·00	1·205	0·795
100	2·00	1·205	0·795

X cc. *M*/12 cysteine hydrochloride in a volume of 50 cc.

0·00	1·00	10·07	0·00
0·20	1·00	8·42	0·165
0·40	1·00	6·81	0·326
0·60	1·00	5·24	0·483
0·80	1·00	3·64	0·643
1·00	1·00	2·08	0·799

Results approximating to the theoretical can be obtained at room temperature by a direct titration with iodine if the cysteine is contained in approximately 80 % acetic acid solution. The presence of this high concentration of acetic acid appears to minimise side-reactions and to ensure oxidation

to disulphide, but because of the low concentration of water, starch cannot be used as an indicator and the end-point must be judged by the first persistent yellow tinge imparted to the solution by excess of iodine.

Derivatives of cysteine.

The cysteine titration curve differs remarkably from that of apparently closely related thiol compounds, *e.g.* glutathione, ergothioneine, simple mercaptans and thiol acids (see below). It was hoped that by studying the reactions of compounds formed by substituting the hydrogen in the carboxyl group of cysteine, the amino-group and finally both groups, some explanation might be found for the peculiar peak at about p_H 2 in the titration curve. Several derivatives of cysteine were accordingly synthesised and their iodine consumptions were studied at different acidities and temperatures.

The first derivatives studied were those in which the carboxyl group was esterified. The ethyl ester hydrochloride of cystine was prepared by passing dry HCl gas into a suspension of cystine in warm absolute alcohol. After boiling on a water-bath for 12 hours, until the cystine had all dissolved, the solution was filtered hot and set in the ice-chest to crystallise. After being twice recrystallised from ethyl alcohol and dried in a vacuum over sulphuric acid, micro-Kjeldahl determinations gave 7·44, 7·47 and 7·68 % of N. Micro-determinations of S gave 17·43 %, of Cl 19·99 and 20·51 %[1]. (Theory for the dihydrochloride, 7·59 % N, 17·4 % S, 19·3 % Cl.)

In a similar manner the *iso*propyl ester was prepared and isolated as the dihydrochloride. After one recrystallisation micro-Kjeldahl gave 7·6 % N; theory requires 7·06 %.

These substances were reduced with zinc in aqueous hydrochloric acid solution immediately before titration, filtered and diluted to volume with oxygen-free water.

A derivative with the amino-group protected was prepared by forming acetylcysteine by a modification of Pirie's [1931] method. The product was practically free from any odour of acetic acid. Analysis, 8·36, 8·38 % N; calc. 8·52 %.

Finally, a compound with both groups substituted, the ethyl ester of acetylcysteine was prepared by acetylation of the ethyl ester of cystine in pyridine. The ethyl ester of diacetylcystine gave on analysis 7·38, 7·58 % N; 16·93, 17·33 % S; (Theory 7·37 % N; 16·84 % S). This was reduced with zinc dust in acid solution just before titration, as in the case of the simple esters.

Effect of substitution in the carboxyl group (ethyl and isopropyl esters of cysteine). Solutions of cysteine ethyl ester are much more stable than cysteine solutions. On standing in an open flask at room temperature for 8 days less than 20 % of the compound in solution was oxidised.

Between p_H 0 and 5·5 there was no variation in the iodine uptake. Above p_H 6 slightly larger amounts of iodine were combined. There is, however, an

[1] We are indebted to Dr Helen Stantial for the micro-analyses reported in this paper.

appreciable temperature effect, as shown in Fig. 1. The *iso*propyl ester behaves similarly.

Effect of substitution in the amino-group (acetylcysteine). The peculiar increase in the iodine consumption of cysteine on direct titration reaches its maximum at p_H 1·8, at which acidity the iodine uptake is almost 50 % greater than in a solution of p_H 0. In the case of acetylcysteine the maximum occurs at p_H 4·5 and is much more pronounced, over 100 % more iodine being required than at p_H 0 (Fig. 4).

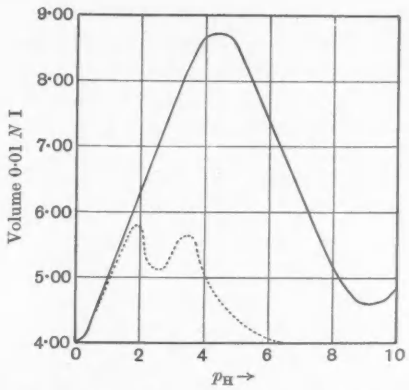


Fig. 4. Iodimetric titration of acetylcysteine at different p_H values: 0·04 millimol. acetylcysteine in 12 cc. buffer; direct titration at 0°. Dotted line is curve for cysteine titrated under similar conditions.

Effect of substitution in both the carboxyl and amino-groups (ethyl ester of acetylcysteine). In the case of the ethyl ester of acetylcysteine, the iodine consumption was practically constant between p_H 0 and 8.

From these results it would appear that an appreciable rise in the curve relating iodine consumption with p_H occurs only if the carboxyl group of cysteine remains free. Esterification eliminates the variations in iodine consumption between p_H 0 and 5, giving compounds similar in their behaviour in this respect to glutathione and to simple thiol acids. The shift of the region of maximum iodine consumption to a less acid range and the augmentation of iodine uptake in the case of acetylcysteine may possibly be related to the fact that on introducing an acyl group into the amino-group its basic properties are weakened, allowing a corresponding increase in the strength of the adjacent carboxyl group.

Glutathione.

One of the early improvements in the estimation of glutathione, based on Tunnicliffe's [1925] iodimetric procedure, was the use of excess iodine in the presence of potassium iodide by Perlzweig and Delrue [1927]. The potassium iodide stabilises the starch end-point by minimising the extent to which side-

reactions occur. Kuhnau [1931] gave a careful review of the biochemical literature on the estimation of this compound, and determined the conditions under which glutathione could be most favourably determined in liver extracts. King and Lucas [1931] showed that at temperatures of 20° and under, crystalline glutathione required theoretical amounts of iodine when titrated at acidities greater than p_H 5. Above this p_H larger amounts were used.

The effect of dilution on the capacity of glutathione for reducing iodine, both on direct and indirect titration, and the marked action of potassium iodide in minimising this dilution error are shown in Table VII. It is noted that

Table VII. *Titration of glutathione at different dilutions.*

3·155 mg. glutathione* in *N*/100 HCl titrated at 10°.

	Direct titration				Indirect
Volume cc.	0·01 *N* I used cc.	End-point correction cc.	0·01 *N* I consumed cc.	0·01 *N* I consumed in presence of KI cc.	0·01 *N* I consumed cc.
1	0·85	—	0·85	—	—
5	0·88	—	0·88	—	0·83
10	0·91	0·02	0·89†	0·84 (1 % KI)	—
20	0·98	0·05	0·93	—	—
30	1·08	0·07	1·01	—	—
50	—	—	—	—	1·31
60	1·30	0·12	1·18†	—	—
100	—	—	—	—	1·47
					0·87 (1 % KI)
120	1·57	0·30	1·27	0·90 (1 % KI)	—
				0·86 (2 % KI)	

* The sample of glutathione used in this experiment was a non-crystalline product prepared in 1930 from yeast by the method of Pirie [1930] and was approximately 20 % in the oxidised form at the time these titrations were made.

† The 10 cc. dilution corresponds roughly to the concentration of glutathione in a 10 % extract of liver, and the 60 cc. dilution corresponds roughly to that in a 1 in 10 trichloroacetic acid filtrate of blood.

in the neighbourhood of the glutathione concentration commonly reported for liver extracts there is approximately an 8 % error in the amount determined by direct titration. In the case of the concentration corresponding approximately with those reported for blood-filtrates there is a 40 % error on direct titration and a 60 % error on indirect titration. These errors would appear to be eliminated by the presence in the solution of 1 % potassium iodide, without suppressing the iodine uptake below the point corresponding to simple oxidation to disulphide. The above titrations were made at p_H 2, but it was also found that the addition of 2 % potassium iodide diminished the iodine uptake almost to the theoretical even when the titration was carried out at neutrality.

Ergothioneine[1] (*the betaine of thiolhistidine*). On account of the occurrence of this sulphydryl compound in blood-filtrates its behaviour towards iodine under various conditions of temperature and acidity was investigated. An approximately *M*/100 solution was prepared by dissolving 33 mg. of the dry

[1] The ergothioneine used in this experiment was kindly supplied to us by Prof. G. Hunter, of the University of Alberta, to whom our thanks are due.

substance in 15 cc. of freshly boiled distilled water. At 0° 1 cc. required 0·892 cc. of 0·01 *N* I by direct titration from a micro-burette; at 20° 0·904 cc. were required, and at 40° 0·924 cc.; the temperature effect, therefore, is almost negligible. That the dilution effect is also very small would appear from the fact that 1 cc. undiluted required 0·904 cc. of 0·01 *N* I, 1 cc. diluted to 10 with distilled water required 0·935 cc. iodine; diluted to 20 cc. it required 0·920 cc. and to 50 cc. 0·890 cc.

Titration of ergothioneine at different hydrogen ion concentrations using glycine, acetate and phosphate buffers, showed that the p_H effect was small on the acid side of neutrality, being almost a straight line between p_H 0 and 6·4. At reactions more alkaline than p_H 7 very much larger amounts of iodine were consumed, and the end-point was transient. By titration of excess iodine with thiosulphate it was found that a total of six times as much iodine was required as that used below p_H 7 (see Table VIII).

Table VIII. *Effect of* p_H *on iodine titration of ergothioneine.*

1 cc. of *M*/100 ergothioneine + 4 cc. buffer titrated at 21° with 0·01 *N* I.

p_H	cc. I	p_H	cc. I
0	0·889	7·0 (first transient end-point)	0·95
1	0·900	7·0 (final end-point)	1·03
2	0·930	7·3 (first end-point)	1·2
3·7	0·945	7·3 (final end-point)	5·9
5·8	0·960	9 (first end-point)	3
6·4	0·950	9 (final end-point by back titration)	5·9
6·4 (without buffer)	0·930		

The presence of added potassium iodide in an ergothioneine solution appeared to have a most marked effect on the amount of iodine consumed. Thus 1 cc. of 0·01 *M* ergothioneine in 5 cc. distilled water required 1·03 cc. 0·01 *N* I, while the presence of 1 % KI depressed the iodine consumption to 0·76 cc. In acid solution the discrepancy was greater: 1 cc. in 5 cc. of *N*/10 HCl required 1·00 cc. 0·01 *N* I and only 0·41 cc. in the presence of 1 % KI. 1 cc. of ergothioneine in 10 cc. of 2·5 % trichloroacetic acid required 0·99 cc. I and the addition of 1 % of KI caused the amount consumed to fall to only 0·20 cc. Under the condition most favourable to titration of cysteine and of glutathione, therefore, ergothioneine reduces only a small fraction of its theoretical equivalent of iodine. This point has recently been considered by Meldrum [1932] and Woodward and Fry [1932] in their study of blood-glutathione determination.

Thiophenols.

In the case of thiophenol and thiocresol the iodine consumption was constant between p_H 0 and 7. At p_H 7·5 a definite increase was noted. The temperature effect was almost nil (see Figs. 1 and 5).

Thio-acids.

Thioglycollic acid and thiolactic acid were chosen as being examples of simple thiol compounds containing a carboxyl group. The marked influence of

temperature when the titration was carried out in N hydrochloric acid is shown in Fig. 1. At 0° the amount of iodine combined corresponded most closely with the stoichiometric relationship $2XSH + I_2 \rightarrow XSSX + 2HI$. The iodine consumption was constant between p_H 0 and 6·5, as shown in Fig. 5.

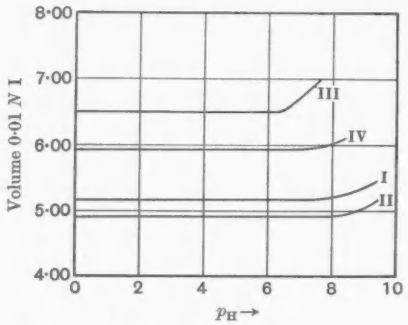


Fig. 5. Effect of p_H on the iodine consumption of thiophenols and thiol-acids. (I) 1 cc. 0·052 M thiophenol, (II) 1 cc. 0·049 M thiocresol, (III) 2 cc. 0·033 M thioglycollic acid, (IV) 2 cc. 0·03 M thiolactic acid.

Summary.

Temperature exerts a marked effect on the amount of iodine combined by cysteine in solution. Extraordinary differences are found in the amounts of iodine consumed at different hydrogen ion concentrations. Esterification of the carboxyl group lessens the temperature effect and almost eliminates the c_H effect. Acetylation of the amino-group, on the other hand, increases the maximum of iodine consumption and alters its position on the p_H curve. The concentration of cysteine in the solution titrated and the concentration of the iodine used have a marked effect on the extent to which oxidation occurs. In the presence of 0·5 % potassium iodide cysteine can be determined quantitatively by indirect iodimetric titration at 0° and N acidity.

Glutathione reduces theoretical amounts of iodine only at an acid reaction (below p_H 5) and at a temperature less than 25°. At high dilution the large error which results on either direct or indirect titration can be eliminated by the presence of potassium iodide in the glutathione solution.

Cysteine in its reactions with iodine is much more markedly affected by temperature, c_H and dilution than is the tripeptide, glutathione, of which it is a constituent.

The dissimilarity of the p_H-iodine consumption curve for liver extract from that for cysteine appears to indicate the absence of appreciable amounts of free cysteine from liver.

The iodine consumption of ergothioneine appears to be only slightly affected by temperature, c_H below neutrality and dilution. The presence of added potassium iodide markedly diminishes the iodine consumption, particularly at acid reactions.

Simple mercaptans may be quantitatively determined by direct titration at room temperature. On the acid side of neutrality the acidity of the medium appears to have no effect on the iodine uptake.

The iodine consumption of thiol acids is markedly affected by temperature, theoretical iodine consumption only occurring at or near 0°. The influence of c_H is not apparent on the acid side of neutrality.

REFERENCES.

Bierich and Kalle (1928). *Z. physiol. Chem.* **175**, 115.
Blanchetière and Melon (1927). *Compt. Rend. Soc. Biol.* **97**, 242.
Gavrilescu (1931). *Biochem. J.* **25**, 1190.
Kennaway and Hieger (1927). *Biochem. J.* **21**, 751.
King, Baumgartner and Page (1930). *Biochem. Z.* **217**, 389.
—— and Lucas (1931). *Biochem. Z.* **235**, 66.
Klason and Carlson (1906). *Ber. deutsch. chem. Ges.* **39**, 738.
Kuhnau (1931). *Biochem. Z.* **230**, 353.
Meldrum (1932). *Biochem. J.* **26**, 820.
Okuda (1923). *J. Sci. Agr. Soc.* (Japan), **253**, 1.
—— (1924). *J. Chem. Soc.* (Japan), **45**, 1.
—— (1925). *J. Biochem.* (Japan), **5**, 201, 207.
Perlzweig and Delrue (1927). *Biochem. J.* **21**, 1416.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1931). *Biochem. J.* **25**, 614.
Rosenheim and Davidsohn (1904). *Z. anorg. Chem.* **41**, 231.
Shinohara (1932). *J. Biol. Chem.* **96**, 285.
Tunnicliffe (1925). *Biochem. J.* **19**, 194.
Turner (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 541.
Uyei (1926). *J. Infect. Dis.* **39**, 73.
Woodward and Fry (1932). *J. Biol. Chem.* **97**, 465.

CCXLVIII. THE PREPARATION OF METHYL ESTERS OF PECTIC ACID.

By HAROLD WILLIAM BUSTON and HOMI RUTTONJI NANJI.

Biochemical Department, Imperial College of Science and Technology, South Kensington.

(*Received November 1st, 1932.*)

It has been generally accepted that the naturally occurring water-soluble pectins are methyl esters of pectic acid, this latter substance, which is insoluble in water, being regarded as containing 4 galacturonic anhydride units, whose carboxyl groups are intact and are involved in the formation of the methyl esters. This conception has been based on the fact that the soluble pectins, on treatment with very dilute alkali in the cold, are rapidly converted into free pectic acid, with liberation of methyl alcohol. Further, the methoxyl-content of samples of soluble pectin prepared from fruits, *etc.* frequently corresponds closely with that required for a tri- or tetra-methyl ester of pectic acid [*cf.* Norris, 1926; Norman, 1928]. So far as can be ascertained, however, no attempt has been made to confirm this view by converting pectic acid into its methyl esters. The accomplishment of this reaction is necessary before the relation between pectin and pectic acid can be regarded as firmly established, for observations have been reported from time to time which suggest that the relationship may not be so simple. For instance, Tutin [1921; 1923] reported the liberation of acetone during treatment of pectin by weak alkali, while Ehrlich and Schubert [1926] consider that acetyl groups are also present.

Experiments have accordingly been made in an attempt to prepare the methyl esters of pectic acid; soluble methyl esters have been obtained and have been found to resemble the natural soluble pectins in all respects. There seems therefore no reason to suggest that any reaction other than hydrolysis of the methyl ester is involved in the formation of pectic acid from pectin.

Similar attempts to prepare other (*e.g.* ethyl) esters of pectic acid were, however, entirely unsuccessful. It appears, in fact, that the methyl derivatives are possibly the only stable esters of pectic acid, and that, if other esters are formed, they undergo ready decomposition, not only with loss of the ester group, but actually with scission of the basal pectic molecule.

Experimental.

Preparation of the methyl esters.

Since the object of the experiments was to methylate only the free carboxyl groups of the pectic acid, and not the hydroxyl groups present in the uronic anhydride residues, it was obviously impossible to adopt any of the methods used in the exhaustive methylation of sugars. The following methods suggested themselves:

(*a*) treatment of calcium pectate with methyl oxalate;
(*b*) treatment of silver pectate with methyl iodide;
(*c*) treatment of sodium pectate with methyl sulphate.

For these experiments, calcium pectate was prepared by precipitating a solution of pectin, after treatment for 4 hours with dilute sodium hydroxide, with calcium chloride solution in the presence of acetic acid. Sodium pectate was prepared by the method of Candlin and Schryver [1928] from the same sample of commercial pectin used by these workers. Silver pectate was prepared by addition of aqueous silver nitrate to a 1 % solution of sodium pectate; the silver compound was precipitated as a clear gel, which was thoroughly washed with cold water, and then with graded strengths of alcohol, and finally dried *in vacuo*. The washing and drying were carried out in weak artificial light, since the compound was found to darken rapidly in daylight. The dry silver pectate was obtained as a cream-coloured powder; the Ag content of different samples varied from 30·3 to 32·1 %, the highest figure being obtained in the case of a sample in the preparation of which a large excess of silver nitrate had been used. Calculated on the basis of the Nanji, Paton and Ling [1925] formula for pectic acid, the Ag content of silver pectate should be 30·3 %. The purity of the samples of calcium and sodium pectate was also checked by ash determinations.

Method (*a*). 1 g. of powdered calcium pectate, suspended in 25 cc. dry methyl alcohol, was heated with 5 g. methyl oxalate for 3 hours in a pressure bottle immersed in boiling water. At the end of this time, the mixture was added to 200 cc. hot water, filtered and the filtrate poured into 500 cc. alcohol. A small gelatinous precipitate was obtained, which, after washing with dilute alcohol and drying, weighed 0·09 g. Its methoxyl content, determined by micro-Zeisel, was 3·4 %. It seemed therefore that some esterification had taken place, but the poor yield and low methoxyl content of the product led to the abandonment of the method.

Method (*b*). A number of attempts were made to prepare the methyl esters by the action of methyl iodide on silver pectate under different conditions; the results are summarised in Table I. Usually, 1–2 g. of dry silver pectate were heated with 10–15 g. methyl iodide in 25 cc. methyl alcohol; at the end of the period of heating, the mixture was added to about 300 cc. water, boiled for a short time, the residue of silver iodide and unchanged silver pectate filtered or centrifuged off and the filtrate treated with excess of alcohol to

precipitate the soluble pectins formed. Considerable difficulty was experienced in removing colloidal silver iodide, the presence of which rendered filtration extremely slow and resulted in contamination of the alcohol precipitate with as much as 25 % of inorganic matter. The most satisfactory method of removing this colloidal material was by adding a few cc. of 5 % potassium bromide and allowing the liquid to stand on the steam-bath for an hour. The silver iodide was thus coagulated; from the clear filtrate, the pectic substance was precipitated as a transparent gel which, when dry, contained only about 3 % ash in most cases.

Table I.

Exp. No.	Treatment of silver pectate	Yield of sol. pectin (ash-free) per g. silver pectate g.	Ash in soluble pectin %	Methoxyl content of sol. pectin (corr. ash) %
1	Boiled with CH_3I under reflux, 8 hours	0·1	—	<3
2	Heated with CH_3I at 96° in pressure bottle, 1 hour	0·35	13*	4·2
3	Heated as above, 4½ hours	0·30	3·5	7·28
4*a*	Heated as above, 12 hours	—	2·3	10·50
4*b*	,,	0·42	6·0	8·60
5	Heated in sealed tube at 100°, 12 hours	—	2·3	5·95
6	Heated as above, 48 hours	—	—	6·0

* Silver iodide incompletely coagulated; boiled with *N*/75 HCl in place of potassium bromide.

From the results recorded above, it appeared that the greatest degree of esterification was produced by heating silver pectate with methyl iodide and methyl alcohol under pressure at 96° for several hours; heating at slightly higher temperatures, or for prolonged periods, resulted in much charring. The products obtained resembled in appearance and solubility the natural soluble pectins; further tests of their identity are described below.

Since a rather wide variation in the methoxyl content of the various products was met with, it was of interest to ascertain whether this was due to the fact that only partial esterification of the carboxyl groups had taken place, or whether substances other than pectins had been formed and carried down in the alcohol precipitate along with the pectins. Accordingly the calcium pectate values for certain of the products were determined; from these a calculation was made of the true methoxyl content of the actual pectic substance present [*cf.* Norman, 1928]. The results (Table II) suggested that even when conditions were apparently most satisfactory there was some production of non-pectin degradation products, possibly hemicellulose-like bodies. As will be seen later, these may in some cases comprise the major part of the products. It may be pointed out that in the case of sample 2, the apparent 25 % of non-pectic material includes the 13 % ash present in the sample; hence the actual amount of organic contaminant is only about 10 %.

Table II.

Sample No.	Methoxyl-content %	Ca pectate value %	MeO, corr. by Ca pectate %
2	3·65	75	4·85
4*a*	10·26	91	11·51
4*b*	8·09	81	9·98
5	5·82	68	8·55

[1]In column 2, the methoxyl content of the sample, uncorrected for ash, is recorded, since the correction applied on the basis of the calcium pectate value will cover this.

Since this sample was prepared by heating only for 1 hour, the low methoxyl value (4·85 %) indicated incomplete esterification, and not extensive decomposition. In the case of sample 5, the prolonged heating of the reaction mixture led to the formation of large amounts of degradation products, reflected in the low calcium pectate value.

With regard to the yield of soluble pectin, in Exp. 4*b* the amount of true pectic substance obtained from 1 g. silver pectate was 0·35 g.; since the theoretical yield of tetramethyl ester per g. of silver pectate is 0·74 g., this represented a yield of approximately 47 %. The solid residue in the pressure bottle was found to contain 0·18 g. unchanged silver pectate.

Method (*c*). A number of attempts to convert sodium pectate into soluble methyl esters by the action of dry methyl sulphate proved entirely unsuccessful. When the substances were heated together at 60–70°, with precautions to exclude moisture, slight darkening of the sodium pectate took place, but on examining the residue (after washing away the methyl sulphate with alcohol), this was found to consist of unchanged sodium pectate. In the presence of moisture, pectic acid was formed from the sodium salt, and this was further attacked by the methyl sulphate (or by sulphuric acid produced by partial hydrolysis thereof) with liberation of carbon dioxide and complete breakdown of the pectin molecule. In no case could any substance resembling pectin be isolated from the mixtures.

Properties of the methyl esters obtained.

As stated above, the appearance and solubilities of the products obtained by the silver pectate-methyl iodide reaction resembled those of the natural soluble pectins. Treatment with lime water produced an immediate precipitate of a typical calcium pectate gel. In the presence of $N/10$ sodium hydroxide, methyl alcohol was rapidly liberated, de-esterification being complete within 15 minutes—a rate comparable with that observed in the case of natural soluble pectin. The soluble pectins were strongly dextrorotatory.

A marked characteristic of the natural soluble pectins of high methoxyl content is their ability to form jellies with sucrose in the presence of traces of acid; among the pectic substances, soluble pectin alone possesses this property [Branfoot, 1929, p. 128]. On this account, sucrose-acid jellies were made from the synthetic methyl esters, and their strengths compared with those

of similar jellies prepared from natural pectin. Jelly strengths were estimated in an apparatus similar to the "pectinometer" of Luers and Lochmüller [1927], but adapted to deal with quantities of 10 cc. of jelly, since the amounts of soluble pectin available would not permit of the preparation of larger quantities. The results are shown in Table III (methoxyl contents shown are corrected on the basis of the calcium pectate value of the sample).

Table III.

Sample	Methoxyl-content %	Jelly strength: 1·5 % pectin g.	Jelly strength: 0·75 % pectin g.
Grape-fruit pectin	11·60	320	—
Commercial citrus pectin*	9·1	270	175
Methyl ester No. 4*a*	11·51	310	180
Methyl ester No. 4*b*	9·98	285	—
Methyl ester No. 5	8·55	180	105

* Pectin from which sodium pectate and silver pectate used in these experiments was prepared.

The results indicated quite clearly that the esters now prepared were comparable in jellying properties with the original natural soluble pectins.

Examination of the figures for methoxyl content of the various products suggested that in each case mixtures of the esters were formed, since none of the figures approximated to the methoxyl-content of a definite di-, tri- or tetra-methyl ester of pectic acid.

Attempted preparation of ethyl esters.

So far as is known, only the methyl esters of pectic acid occur naturally [von Fellenberg, 1918]. In order to see whether other esters resembled the methyl derivatives in their properties, an attempt was made to prepare the ethyl esters by heating silver pectate and ethyl iodide together under the same conditions as were found successful in producing methylation.

During the reaction, the mixture was observed to become very dark in colour. The aqueous extract after the reaction gave no precipitate with alcohol, but on evaporation left a brownish gum, which gave a strong reduction with Fehling's solution. Since an examination of the solid residue in the pressure bottle showed this to consist almost entirely of silver iodide, with a little unchanged silver pectate, it seemed likely that the reaction between the silver pectate and ethyl iodide had actually taken place with formation of ethyl pectates, and that it was the esters which underwent subsequent decomposition, with formation of the gummy reducing substances. This view was further supported by the observation that pectic acid itself was not appreciably destroyed by heating for some hours at 96° with ethyl iodide, alone or in the presence of absolute alcohol. It may be suggested, tentatively, that the introduction of a heavier group into the pectic molecule (even the substitution of C_2H_5— for CH_3—) results in an instability of the pectic ring, and its consequent breakdown to simpler substances. In this connection one may recall

the comparative ease with which pectins have been found to break down into hemicellulose-like bodies, sugars, *etc.* [*cf.* Candlin and Schryver, 1928; Linggood, 1930; Norman and Norris, 1930].

Summary.

1. Pectic acid is readily converted into its methyl esters by heating silver pectate with methyl iodide under slight pressure. The products resemble the natural soluble pectins in all respects.

2. There is no reason to suppose that the soluble pectins are other than simple methyl esters of pectic acid.

3. The ethyl esters of pectic acid are apparently unstable, undergoing extensive decomposition under the experimental conditions used in their preparation.

REFERENCES.

Branfoot (1929). A critical and historical study of the pectic substances of plants.
Candlin and Schryver (1928). *Proc. Roy. Soc. Lond.* B **103**, 365.
Ehrlich and Schubert (1926). *Biochem. Z.* **169**, 13.
von Fellenberg (1918). *Biochem. Z.* **85**, 45.
Linggood (1930). *Biochem. J.* **24**, 262.
Luers and Lochmüller (1927). *Kolloid Z.* **42**, 154.
Nanji, Paton and Ling (1925). *J. Soc. Chem. Ind.* **44**, 253 T.
Norman (1928). *Biochem. J.* **22**, 749.
—— and Norris (1930). *Biochem. J.* **24**, 402.
Norris (1926). *Biochem. J.* **19**, 993.
Tutin (1921). *Biochem. J.* **15**, 494.
—— (1923). *Biochem. J.* **17**, 83.

APPENDIX.

Small-scale "pectinometer."

The small-scale "pectinometer" used in testing the strengths of the sugar-pectin jellies in the above work was devised and made by our laboratory assistant, Mr G. Howkins. It was designed to deal with one-tenth the amount of jelly used in the ordinary pectinometer, *i.e.* 0·15 g. pectin in 10 g. jelly.

The vessel in which the jelly is allowed to set consists of a piece of thin copper tubing, 6·5 cm. long and 2·4 cm. internal diameter closed at the bottom. Into the sides of this are drilled and soldered 8 pieces of brass wire (S.W.G. 16), projecting 5 mm. into the tube; these are arranged in two circles, the lower being 1 cm. and the upper 1·5 cm. from the bottom of the tube, and serve to hold the jelly firmly in the tube. Along the axis of the tube is fixed a piece of similar wire,

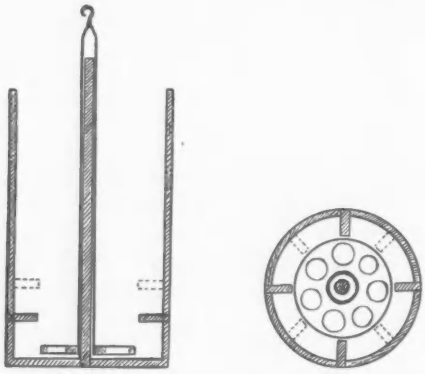


Fig. 1. Small-scale "pectinometer"; arrangement of vessel in which jelly is "set," and device for "tearing" jelly.

8 cm. long, to serve as a guide for the jelly-tearing device. This latter consists of a thin copper tube, 10 cm. long and as narrow as possible, which just slides easily over the guide-wire; to its lower end is fixed a circle of brass, 12 mm. diameter, perforated with 7 holes at its periphery. The upper end of the inner tube carries a hook, to which is attached a fine chain, passing over two pulley-wheels to a counterpoised balance-pan, as in the ordinary pectinometer.

In making the test, sufficient of the pectin sample to yield 0·15 g. calcium pectate is dissolved in 10 cc. of 0·2 % tartaric acid solution, together with 6 g. sucrose. The mixture is boiled in a 30 cc. beaker until the weight of the mixture is reduced to 10 g. (By means of a preliminary test, a mark is made on the beaker to indicate this point.) The boiling is completed within 15 minutes. The hot mixture is transferred immediately to the setting-tube of the pectinometer, in which it forms a layer about 2 cm. deep. The subsequent procedure in estimating the strength of the jelly is exactly the same as in the case of the ordinary pectinometer. The apparatus has proved sufficiently satisfactory, giving comparable results within about 5 %.

CCXLIX. THE RAT UNIT OF TESTICULAR HORMONE.

By VLADIMIR KORENCHEVSKY, MARJORIE DENNISON and ALICE KOHN-SPEYER.

From The Lister Institute, London.

(*Received November 1st, 1932.*)

In previous papers [Korenchevsky, 1932, 1, 2; Korenchevsky, Dennison and Schalit, 1932] a detailed description has been given of the method of assay of testicular hormone, using rats as the experimental animals, and a new rat unit was suggested for use in the assay of this hormone.

In the present investigation a test was made of the method of assay and the suitability of this rat unit, and a comparison of the size of the rat unit (RU) with that of the capon unit (CU).

Technique.

Experiments were performed on 42 rats belonging to 8 litters. The technique of the assay, described previously, was followed strictly. The ages of the rats at castration, the number of days after castration before the injections were started, and the weights of the rats at the end of the experiments are given in Table I.

Table I. *Age and average final weights of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Age at castration (days)	No. of days after castration injections started	Weight of rats (g.)					
				Control rats	Rats injected with				
					1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
1	6	24	50	221	230	234	—	—	—
2	7	24	50	197	182	204	—	—	—
3	5	27	43	166	184	182	—	—	—
4	3	29	32	174	—	—	193	—	—
5	6	27	32	217	—	—	—	218	210
6	5	27	32	194	—	—	209	—	—
7	5	22	32	192	—	—	—	191	—
8	5	25	75	343	—	—	—	387	—

A well-purified testicular hormone, isolated from urine, belonging to one and the same batch, prepared by Messrs Schering Ltd., was used for the injections[1]. The hormone was dissolved in oil and its strength was stated to

[1] We wish to express our thanks to Messrs Schering Ltd. for kindly supplying this preparation.

be equal to 25 capon units per cc., as standardised in the Schering Laboratories. We have not re-tested the preparation on capons, and in the following its strength will be designated in capon units according to Schering's standardisation.

For the injection of small doses, sufficient of the original preparation for the day's injection was diluted, each morning, with sesame oil under aseptic precautions. For the injection of 5·0 and 7·5 CU daily the undiluted original preparation was used. The daily dose was injected subcutaneously twice a day, in half doses, at about 10 a.m. and 5 p.m. for 7 consecutive days.

After the morning injection the remaining doses for the evening injection were covered with sterilised glass lids and placed in the cold store. In no case were indurations or any local reactions noticed, nor was there depression of the animals or of their appetite.

All the litters were castrated before sexual maturity at the age of 22 to 29 days.

As can be seen from Tables II to VI, only the first 7 litters were used in the standard test. These were chosen intentionally so that the number of days after castration was between 32 and 50 days and therefore the weights of the rats did not differ greatly—the average weights at the end of the experiment being between 166 and 234 g.

Litter 8, which was about double the weight of the other litters (see Table I), was chosen to show the correlation between the dose and the weight of the rat. The results of the injections into the rats of this litter are discussed below in a separate paragraph and are not included in Tables II to VI in order to avoid confusion. The supply of testicular preparation was unfortunately insufficient for the treatment of more than one litter of large rats.

The influence on prostate with seminal vesicles.

The results obtained are summarised in Table II. In previous work [Korenchevsky *et al.*, 1932] experiments showed that the variations in the increase of prostate with seminal vesicles were not large in rats belonging to different litters injected at a similar period after castration with the same dose of the same testicular extract, provided the rats were operated on at the same time in relation to sexual maturity. The same results were obtained in the present experiments, as can be seen from a comparison of the percentage increase of prostate with seminal vesicles of rats injected with the same dose but belonging to different litters (Table II). The variation was considerable in only one group of the rats injected with one CU. However, it must be remembered that this, the smallest dose of one unit, would give an increase comparatively close to the limit of physiological variations [Korenchevsky, 1932, 2] of the weight of prostate with seminal vesicles. In a standardisation using the smallest dose, therefore, it is sometimes difficult in a biological test to rely on an increase of 20 or 30 mg. in the weight of the organs, since physiological variation of a few mg. will influence the results obtained considerably.

Table II. *Influence on prostate with seminal vesicles.*

The average weights, actual and calculated, per 200 g. of body weight, of the prostate and seminal vesicles of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage increase in weight				
		1 cu	1·5 cu	2·5 cu	5·0 cu	7·5 cu	1·0 cu	1·5 cu	2·5 cu	5·0 cu	7·5 cu
					A. Actual weights.						
1	64	97	105	—	—	—	51·5	64·0	—	—	—
2	62	81	96	—	—	—	30·6	54·8	—	—	—
3	71	100	117	—	—	—	40·8	64·8	—	—	—
4	56	—	—	120	—	—	—	—	114·1	—	—
5	55	—	—	—	160	219	—	—	—	190·8	298·0
6	56	—	—	111	—	—	—	—	98·2	—	—
7	53	—	—	—	154	—	—	—	—	190·6	—
Av.	**60**	**93**	**106**	**116**	**157**	**219**	**41·0**	**61·2**	**106·2**	**190·7**	**298·0**
					B. Weight calculated per 200 g. body weight.						
1	58	85	90	—	—	—	46·5	55·2	—	—	—
2	63	90	94	—	—	—	42·8	49·2	—	—	—
3	87	109	129	—	—	—	25·3	48·3	—	—	—
4	64	—	—	124	—	—	—	—	93·8	—	—
5	51	—	—	—	146	212	—	—	—	186·2	315·5
6	58	—	—	106	—	—	—	—	82·9	—	—
7	55	—	—	—	162	—	—	—	—	194·5	—
Av.	**62**	**95**	**104**	**115**	**154**	**212**	**38·2**	**50·9**	**88·4**	**190·4**	**315·5**

To correct this at least 3 litters should be used and the averages can then be considered reliable and, as can be seen from the tables, this procedure gives satisfactory results. It would be still better to use for the standardisation doses of about 2 or 3 units when small physiological variations would be relatively less important.

In the previous experiments a close correlation of direct proportion was obtained between the increase in the dose of the hormone injected and the average increase of the prostate with seminal vesicles.

It can be seen from Tables II and V that the same correlation was found in the present experiments. At the same time, when the weights of the rats are so nearly the same as was the case in our experiments, the average percentage changes for the actual weights are very similar to those calculated for the same unit of rat's weight. In both our previous and our present experiments the unit of 200 g. of body weight was chosen, since this is the average weight of the rats most often used for the experiments.

The influence on the penis.

As was stated in the previous paper, it was found that the penis responds to the injection of testicular hormone more slowly and in a less regular way than the prostate with seminal vesicles. However, as is shown in Tables III and V, the present experiment corroborates our previous statement, that, for the assay, the changes in the penis give an extra proof of the activity and strength of the preparation tested.

Table III. *Influence on the penis.*

The average weights in mg., actual and calculated per 200 g. of body weight, of the penis of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage increase in weight				
		1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU	1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
					A. Actual weights.						
1	82	97	98	—	—	—	18·3	19·5	—	—	—
2	67	79	80	—	—	—	17·9	19·4	—	—	—
3	67	80	103	—	—	—	19·4	53·7	—	—	—
4	67	—	—	104	—	—	—	—	55·2	—	—
5	64	—	—	—	110	137	—	—	—	71·9	114·0
6	70	—	—	111	—	—	—	—	58·6	—	—
7	66	—	—	—	110	—	—	—	—	66·6	—
Av.	**69**	**85**	**94**	**108**	**110**	**137**	**18·5**	**30·9**	**56·9**	**69·3**	**114·0**
					B. Weights calculated per 200 g. body weight.						
1	74	85	84	—	—	—	14·8	13·5	—	—	—
2	68	87	78	—	—	—	27·9	14·7	—	—	—
3	82	87	113	—	—	—	6·1	37·8	—	—	—
4	77	—	—	108	—	—	—	—	40·3	—	—
5	59	—	—	—	100	131	—	—	—	69·5	122·0
6	73	—	—	100	—	—	—	—	37·0	—	—
7	69	—	—	—	115	—	—	—	—	66·7	—
Av.	**72**	**86**	**92**	**104**	**108**	**131**	**16·3**	**22·0**	**38·7**	**68·1**	**122·0**

Table IV. *Influence on the thymus.*

The average weights of the thymus (mg.) calculated per 200 g. body weight of the rats in each group of each litter and their percentage decrease after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage decrease in weight				
		1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU	1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
1	508	398	410	—	—	—	21·6	19·3	—	—	—
2	644	508	529	—	—	—	21·1	17·9	—	—	—
3	569	564	525	—	—	—	0·9	7·7	—	—	—
4	639	—	—	611	—	—	—	—	4·4	—	—
5	850	—	—	—	616	607	—	—	—	27·5	28·6
6	702	—	—	666	—	—	—	—	5·1	—	—
7	862	—	—	—	734	—	—	—	—	14·8	—
Av.	**682**	**490**	**488**	**639**	**675**	**607**	**14·5**	**15·0**	**4·8**	**21·2**	**28·6**

In the most recent paper [Korenchevsky, 1932, 2] it was stated that the weighing of the dissected penis is more accurate if it is fixed in Bouin's fixative before weighing (*i.e.* the penis should be treated in the same way as the other organs). In this way more regular changes after injection were obtained in the penis.

From Tables III and V it is clear that, although the response of the penis to the injections was less sensitive, this organ, like the prostate with seminal vesicles, showed on the average changes in weight in direct proportion to the dose injected; for each RU or CU an increase was obtained in the weight of the

Table V. *The relation between the rat unit and the capon unit of testicular hormone.*

Dose of CU injected	Average % increase in weight: Obtained	Average % increase in weight: Expected	% increase obtained per CU: With each dose	% increase obtained per CU: Average (max. % deviations in brackets)	No. of rat units in dose
Prostate + seminal vesicles:		A. Actual weights.			
1·0	41·0	—	41·0		1·03
1·5	61·2	61·5	40·8	40·4	1·53
2·5	106·2	102·5	42·5	(+ 5·2 / − 5·7)	2·66
5·0	190·7	205·0	38·1		4·77
7·5	298·0	307·5	39·7		7·45
Penis:					
1·0	18·5	—	18·5		—
1·5	30·9	27·8	20·6	18·2	—
2·5	56·9	46·3	22·8	(+25·3 / −23·6)	—
5·0	69·3	92·5	13·9		—
7·5	114·0	138·8	15·2		—
		B. Weights calculated per 200 g. body weight.			
Prostate + seminal vesicles:					
1·0	38·2	—	38·2		0·96
1·5	50·9	57·3	33·9	37·5	1·27
2·5	88·4	95·5	35·4	(+12·3 / − 9·6)	2·21
5·0	190·4	191·0	38·1		4·76
7·5	315·5	286·5	42·1		7·89
Penis:					
1·0	16·3	—	16·3		—
1·5	22·0	24·5	14·7	15·3	—
2·5	38·7	40·8	15·5	(+ 6·5 / − 11·1)	—
5·0	68·1	81·5	13·6		—
7·5	122·0	122·3	16·3		—

penis of about 18 % (actual weight) or 15 % (when calculated per 200 g. of body weight) as compared with that of the controls.

We would, therefore, advise the use of the changes in the penis as a rough extra proof of the activity and strength of the hormone. In the case of the penis the weights calculated per 200 g. of body weight gave closer results than the changes in the actual weights (Table V).

Influence on the thymus.

The changes observed in the thymus after the injection of testicular hormone are given in Table IV. Since these changes were small, to economise space only the weights calculated per 200 g. of rat's weight are given.

The decrease in the thymus after the injection of the extract used was in most cases small, and in one case (litter 3, dose 1 CU) even negligible; also the correlation of direct proportion between the increase in the dose injected and the degree of decrease is not clearly shown. In the previous experiments similar changes were noted. Nevertheless, in spite of the unsuitability of these changes for standardising the hormone, we would suggest that they be noted during an assay of a preparation as indicating the activity of that preparation on the thymus.

The influence on fat deposition, adrenals, thyroids and hypophysis.

As was found in previous experiments, seven days of injection is too short a period for the production of marked changes in these organs and in fat deposition. For this reason only the average figures of the results obtained with each dose are given (Table VI).

Table VI. *Average percentage changes in weight (actual and per 200 g. of rat's weight) of retroperitoneal fat, adrenals, thyroid and hypophysis of the injected rats compared with those of the control rats.*

	Rats injected with				
Organs	1·0 CU	1·5 CU	2·5 CU	5·0 CU	7·5 CU
Retroperitoneal fat:					
Actual weight	+37·4	+37·0	+13·3	−14·6	−15·9
Per 200 g. rats weight	+34·2	+39·0	+ 6·5	−14·5	−12·4
Adrenals:					
Actual weight	− 1·1	+ 2·1	− 8·2	− 9·4	−16·7
Per 200 g. rats weight	− 4·3	− 5·0	−16·0	−10·0	−13·2
Thyroid:					
Actual weight	+ 7·6	+ 7·6	+ 6·3	+ 9·0	+10·9
Per 200 g. rats weight	+ 4·1	+ 0·3	− 1·5	+ 8·5	+13·4
Hypophysis:					
Actual weight	− 1·2	− 5·2	− 9·4	+ 2·8	− 8·5
Per 200 g. rats weight	− 2·6	−11·2	−19·4	+ 1·1	− 6·8

It has already been stated [Korenchevsky, 1932, 1, p. 420] that, in order to be certain that the changes found after injection are outside the limits of physiological fluctuations, the increase in weight of the thyroids should be not less than 40 %, while for the decrease in weight of the adrenals, hypophysis and fat deposition an even larger figure should be allowed. The changes obtained in the present experiment are therefore too small to permit of any definite conclusions. We would like to note, however, that, though small, there was, in most cases, an increase in the weights of the thyroids and a decrease in the weights of the adrenals and hypophysis, that is, there was a tendency for the organs changed by castration to return to normal.

One more point must be mentioned here, that in castrated rats a period of 1 or 2 months after castration is not sufficient to produce maximum changes in the above organs [Korenchevsky, 1931, 2]. It is therefore necessary, in testing the influence of testicular hormone on these organs, to choose rats which have been castrated not less than 3 months, preferably more, before the test. For the same reasons the changes in fat deposition were also small, while the physiological variations in fat deposition were greater than in any of the organs which have been mentioned [Korenchevsky, 1932, 1]. However, it is noteworthy that, with small doses (1·0 or 1·5 CU) fat deposition increased and with large doses (5 or 7·5 CU) decreased. The intermediate dose of 2·5 CU produced a small increase in fat deposition. If these results are not accidental, they may indicate that metabolism is differently influenced by small and by large doses of testicular hormone.

The rat unit and its relation to the capon unit.

It must be emphasised that, since we did not re-test the capon unit of the preparation used, this unit was taken to be as was stated by Messrs Schering. Up to the present time the method of assay of testicular hormone by using capons has not been standardised and therefore the capon unit may vary in different laboratories.

. With reference to the disadvantages of the capon unit, which have already been discussed [Korenchevsky, 1932, 1, p. 413], it is necessary to add that Womack and Koch [1932, p. 275] have established that the urine of pregnant and of normal women yields "the same amount of comb growth-stimulating material as urine from normal mature men." Kabak [1931] arrived at a similar conclusion. Womack and Koch (p. 276) state that "we have present in urine from pregnant and normal mature women, a substance very similar to or identical with the testicular hormone[1] but that it is different chemically in some respects and acts differently physiologically from the female sex hormone which produces oestrus in the castrated female. The actual source and function of this hormone is at present a mystery." It is important that the authors failed to obtain this hormone from the urine of an ovariectomised woman, which would indicate that the ovaries are the source of this hormone. The specificity of the comb test, therefore, becomes doubtful in several instances and in any case it seems that for the assay of testicular hormone it should be used simultaneously with, if not replaced by, a test on the sexual and other organs of castrated mammals. Taking this into consideration it is necessary to discuss the results of our present experiments with reference to the suitability of the method of assay and of the rat unit as these have been described in our previous and present papers.

In order to answer this question, the results are presented in Table V so as to show the relation between the capon and rat units and the suitability of the latter. It is clear, from the second and third columns of this table, that the increase in weight of the prostate with seminal vesicles obtained in the experiment is very close to that theoretically expected, since there is a direct relationship between the increase in the amount of hormone injected and the increase in the weights of the prostate with seminal vesicles. The fourth and fifth columns show that the percentage deviations from the average increase, corresponding to 1 CU, were not more than 6 % for the actual figures or 12 % when calculated per 200 g. of rat's weight.

This may be considered quite satisfactory for a biological test. The figures in the sixth column of the table were obtained by dividing "% increase obtained" (2nd column) by 40, since the suggestion for the rat unit was the increase in weight of the prostate with seminal vesicles by 40 % as compared with those of the uninjected control rats. As can be seen from the table, the rat units were nearly equal to the capon units. They are more nearly so if we

[1] As far as can be understood from the paper the only test made was that of comb growth in capons.

take the average figure: thus the sum of the doses in CU (column 1) is 17·50 CU, with which corresponds (column 6) 17·44 RU obtained by changes in the actual weight of the prostate with seminal vesicles, or 17·09 RU, obtained by the changes per 200 g. of body weight. From this, in the batch tested, 1 CU corresponds to 0·997 RU (actual weights) or 0·977 RU (per 200 g. of body weight).

Therefore, using our method of assay and definition of the rat unit, this latter proved unexpectedly to be nearly equal to the capon unit (as assayed in the Schering Laboratory).

Precautions necessary in assaying the rat unit.

1. All the precautions and rules detailed in the previous papers are confirmed.

2. *The influence of the difference in weight of the rats.* Since the rats must be castrated before the 30th day after birth, and since the weight of the prostate with seminal vesicles remains constant (per unit of body weight) from the end of the first month after castration [Korenchevsky, 1932, 2], the rats seem most suitable for the assay at the age of about 2 months. A longer period is inconvenient since it means waiting longer until the animals are ready for the assay. In our present experiments we used, without appreciable change in the results, rats castrated 32 to 50 days before the experiments were started. This margin of about 18 days is convenient since even in large Institutions, having their own breeding rooms, it is difficult to obtain at short notice the necessary number of litters of a special age.

The amount of the testicular hormone preparation available was unfortunately only sufficient for the performance of one experiment (on litter No. 8) for studying the influence of the same dose of hormone on rats weighing about twice as much as litters 5 and 7. The weights of the rats of these litters and their ages are given in Table I. The daily dose injected was 5 CU. The results on litter 8 are given in Table VII and, as can be clearly seen, the increase in weight of the prostate with seminal vesicles was considerably less than that in litters 5 and 7 injected at the same time with the same dose, but more than in litters 4 and 6 injected with half the dose (2·5 CU).

This indicates that:

(1) if the same dose of the hormone is injected into rats of different weights, the influence will be less in heavier rats;

(2) a strict direct proportion does not exist between the weight of the rat and the effect of the dose injected per unit of body weight, for in this case the increase of prostate with seminal vesicles instead of 154 % (actual weight) and 123 % (per 200 g. body weight) would be equal to that of the rats injected with 2·5 CU, *i.e.* about 106 % and 88 % respectively, since the weight of litter 8 was about twice that of litters 4 and 6.

Since, however, the experiment was performed on only one litter more experiments must be made before drawing a definite conclusion.

Table VII. *Influence of injections of 5 cu per day on average weight of organs of rats of litter 8 as compared with that of non-injected litter-mates.*

	Prostate + seminal vesicles		Penis		Thymus		Retroperitoneal fat	
	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual g.	Per 200 g. body weight g.
Control rats	87	51	88	51	480	278	17·92	10·51
Injected rats	221	114	157	81	484	250	18·28	9·46
% change in weight of organs	+154	+123	+ 78·4	+58·8	+ 0·8	− 10·1	+2·0	−10·0

	Adrenals		Thyroid		Hypophysis	
	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.
Control rats	66·3	38·5	28·1	16·4	11·5	6·7
Injected rats	63·8	32·9	35·8	18·6	14·7	7·6
% change in weight of organs	− 3·8	−14·5	+27·4	+13·4	+27·8	+13·4

3. The dissection of the very atrophied prostate and seminal vesicles from the surrounding fat tissue is a rather delicate task and needs some training. It must be performed with the utmost accuracy, using, if necessary, a dissecting lens, and since these organs in castrated animals weigh only from 50 to 70 mg. the error in leaving a little fat tissue or removing a little of the prostatic tissue might influence the results considerably. At the same time accuracy must not interfere with rapid dissection as the organs must not be allowed to dry in the air, thus creating another source of error.

The accurate removal of fat tissue from all organs is roughly indicated by their sinking at once to the bottom of the vessel containing the Bouin's fixative. If any organ floats it must be removed from the fixative and the fat tissue left unnoticed removed.

For dissecting the hypophysis a very sharp small scalpel should be used.

4. The hormone is usually dissolved in oil and during injection it is necessary to push the needle into the subcutaneous tissue as far as possible from the point of entrance into the skin by pressing the bulb of the needle forward against the skin. In rats, if this is not done, about 15 to 30 minutes after the injection the oil begins to leak out and the loss in this way might be considerable. As a routine our rats are inspected about 45 minutes after the injections and any leakage is noted in the records.

5. The experience of Institutes breeding their own stocks of rats has shown that even with the best care it is not possible to prevent the occurrence of single cases of pneumonia among the rats. Rats suffering from pneumonia, especially those failing to grow or losing weight, must be discarded or the

results obtained with these rats must be taken with reservation, since pneumonia interferes with the influence of the injection of testicular extract. At *post mortem*, therefore, the lungs of the rats must always be examined for pneumonia.

Disadvantages of the method.

For each assay new animals must be operated on and chosen with reference to their age at castration and at the beginning of the experiment.

Advantages of the method.

1. The method of assay is accurate, precise in end-point reading, quick and simple, since it only requires the weighing of the organs and not a microscopic examination.

2. The data obtained give a direct indication of the changes in the secondary sexual organs of the male, *i.e.* the method is specific for the male hormone.

3. The method, when used for longer periods, gives an indication of the changes in most of the other organs and functions which are influenced by castration and usually, even after only 7 days, those in the thymus.

4. In order to determine the number of rat units contained in the dose injected at least 3 litters, preferably more, are injected with the same amount of any reasonable dose of the active material. Since the correlation between the increase of the dose of the injected hormone and the average degree of increase of prostate with seminal vesicles is one of direct proportion, the quotient of division of the average increase of the prostate and seminal vesicles by 40 will be the number of rat units injected (see Table V). In our experiments a test, from this point of view, was made only of doses from 1·0 to 7·5 RU. Whether the direct proportional correlation will remain true for much higher doses has yet to be investigated.

Summary.

1. Using 42 castrated rats belonging to 8 litters a test was made of the method of assay of testicular hormone and the suitability of the rat unit, as defined in previous papers, and a comparison was made betwen the rat unit and the capon unit.

2. From the results obtained, both the method used and the suggested rat unit seem to be satisfactory for the assay of testicular hormone.

3. The rat unit suggested is the minimum daily dose, which, when injected twice a day in half doses during 7 consecutive days, into at least 3 litters of rats castrated before the 30th day after birth, will produce on the average an increase of 40 % in the weight of the prostate with seminal vesicles as compared with the average weight of these organs in the uninjected litter-mates. For the assay it seems most convenient to use rats about 30 to 50 days after castration.

4. The rat unit proved to be nearly equal to the capon unit (as assayed in the Schering Laboratories).

5. On the average, a correlation of direct proportion was found between the increase of the dose, from 1 to 7·5 RU, and the degree of average increase in the weight of prostate with seminal vesicles and, less sensitively, of the penis.

6. For each RU or CU the increase in weight of the penis was about 18 % (actual weight) or 15 % (when calculated per 200 g. of body weight) as compared with that of the controls.

7. The response of the thymus to the injections of the hormone was variable and less sensitive.

8. Although, for assaying testicular hormone, a rat unit is suggested which is based on the more sensitive response of the prostate with seminal vesicles, it is also useful to note the changes in weight of the penis and thymus as supplementary proofs of the activity of the preparation.

9. The precautions which are necessary for obtaining accurate results in the assay are detailed, with special reference to the weight and age of the rats used for assay.

10. The disadvantages and advantages of the proposed method of assay and of the rat unit are discussed.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due.

REFERENCES.

Kabak (1931). *Endokrinologie*, **9**, 84.
Korenchevsky (1932, 1). *Biochem. J.* **26**, 413.
—— (1932, 2). *Biochem. J.* **26**, 1300.
—— Dennison and Schalit (1932). *Biochem. J.* **26**, 1306.
Womack and Koch (1932). *Endocrinology*, **16**, 273.

CCL. THE REACTIONS OF LEAD COMPOUNDS WITH SERUM AND SERUM-MODELS[1].

By MAURICE JOWETT.

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received November 4th, 1932.*)

The reactions of lead compounds with serum are of interest, since they determine largely the fate of lead entering the body.

The first fairly direct evidence that lead ions react with the phosphate ions of serum was apparently obtained by Aub and his co-workers [1924], who found that haemolysis in hypotonic salt solutions was lessened by addition of soluble lead salts. Lead salts previously mixed with serum had no such effect. At the concentration present in serum, phosphates were the only substances found to have the same power of inhibiting the action of lead as had serum itself.

Scremin [1925] added solid lead iodide, carbonate and sulphate to serum, and found phosphate in the sedimented precipitates.

Brooks [1927] ultrafiltered serum and aqueous solutions after adding small quantities of lead acetate or of a suspension containing metallic lead and lead hydroxide. The lead in the mixtures was not filterable, and some of the phosphate had ceased to be filterable. The observed mean ratios of phosphorus atoms removed from solution to lead atoms added are given in Table I.

Table I. *Data of Brooks.*

	Filtered	P/Pb
Lead acetate added to phosphate + NaCl	At once	0·62
Lead suspension added to phosphate + NaCl	After 7 days	0·62
Lead acetate added to Ringer's solution	At once	0·69
Lead suspension added to Ringer's solution	After some days	1·0
Lead acetate added to cow serum	After some days	1·1
Lead suspension added to cow serum	After some days	1·1

These results were interpreted by Brooks as indicating formation of $PbHPO_4$ or $Pb_3(PO_4)_2$, which would require ratios of 1·00 or 0·67.

Bischoff and Maxwell [1928] made similar experiments, including however analyses for calcium, and found that calcium also became unfilterable. Figures calculated from their experiments are given in Table II. They show that calcium precipitates with the lead, except in acid solutions.

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell).

Table II. *Data of Bischoff and Maxwell.*

	Ca/Pb	P/Pb	P/Ca + Pb
Lead acetate added to Ringer's solution	0·28†	0·67	0·52
Lead hydroxide added to Ringer's solution	0·73	0·90	0·52
Lead acetate added to phosphate + $CaCl_2$*	0	0·70, 0·63	—
Lead acetate added to rabbit serum	1·03	1·21	0·60
Lead acetate added to rabbit serum	0·92	1·39	0·72
Lead acetate added to rabbit serum	0·92	1·19	0·59
Lead acetate added to pig serum	0·94	1·37	0·71
Mean of serum experiments	0·95	1·29	0·655

* The final p_H was <6·0. In the other experiments it was >7·0.
† The low value is due to a shortage of calcium in the solution.

In serum the data, as interpreted by the authors, would indicate that probably $Pb_3(PO_4)_2.Ca_3(PO_4)_2$ is formed, since this would require the ratios in the table to be 1·00, 1·33 and 0·67. Bischoff and Maxwell assume, however, that only filterable calcium reacts with phosphate, although they themselves show that non-filterable calcium can disappear by reaction with oxalate. If we assume instead that the partition of calcium between the forms initially present is unchanged by addition of lead, we can derive from their data the ratios 1·33, 1·29 and 0·56, which no longer approximate to the requirements of $Pb_3(PO_4)_2.Ca_3(PO_4)_2$.

The exact product of the addition of lead salts to serum is therefore still uncertain, and the present work was undertaken to obtain further information on its nature, and on the rates of the reactions, which are of toxicological interest. It seemed improbable that the ultrafiltration method already used would yield much more of interest, and the author has employed a manometric method, which is less specific but more suited to measuring rates of reaction. The lead electrode has also been of value.

A less direct method of attack follows from the general tendency for the formation in precipitations of the least soluble salt. Measurements of the solubility of lead carbonate will be given before other salts are considered.

The solubility of lead carbonate.

Measurements of the solubility of lead carbonate in aqueous solutions at 37·5° were made by the manometric methods of Warburg [1926]. As the results are not very accurate, they are reported briefly.

2–4 cc. of dilute solutions of lead acetate, containing sometimes a little added HCl or $NaHCO_3$, were shaken in manometric vessels in the presence of nitrogen containing about 5 % CO_2. Lead carbonate precipitates according to the equation

$$Pb^{++} + CO_2 + H_2O \rightarrow PbCO_3 + 2H^+.$$

After some hours excess of HCl is added from a separate compartment in the vessel; the lead carbonate redissolves, and the CO_2 previously absorbed is evolved. The increase in pressure tells us the amount of $PbCO_3$ that had precipitated—some 30–40 % of the total lead—and hence the amount of Pb^{++}

left in solution. We can also calculate the amount of H^+ that has been liberated; this reacts almost completely with acetate ion to give acetic acid. From the ratio of acetic acid to acetate in the system we obtain the p_H, which in turn regulates the concentration of $CO_3^=$. The solubility product of lead carbonate can therefore be calculated from one pressure change, provided that we know the initial composition of the solution, the CO_2 content of the gas mixture, and the various equilibrium constants and activity coefficients concerned. The presence of H^+ and HCO_3^- in the solutions has to be allowed for in the calculations, which are made by successive approximations.

Experiments were also made in the presence of a suspension of lead carbonate. After some hours' shaking $NaHCO_3$ is added instead of HCl, and the Pb^{++} left in solution is estimated by the gas change in the reaction:

$$Pb^{++} + 2HCO_3^- \rightarrow PbCO_3 + CO_2 + H_2O.$$

Similar values were obtained by this method, with which equilibrium between solid and solution should be attained more rapidly. This indicates that equilibrium is probably attained by both methods.

The calculated solubility products are given in Table III. The solubility product S is defined by the relation

$$-\log_{10} S = pS = p\mathrm{Pb}^{++} + p\mathrm{CO}_3^=,$$

in which the ionic terms are activity terms[1].

Table III. *Solubility product of $PbCO_3$ at 37·5°.*

Ionic strength	pS
0·020	11·91
0·019	12·00, 11·88, 12·00, 12·00
0·017	12·00
0·012	12·18, 12·23, (12·02)
0·009	12·24, 12·23, (11·64), 12·23, 12·24
0·008	12·22, 12·24
0·005	(12·42)
0·004	12·16, 12·22

From the values at the lower ionic strengths we take a mean value 12·22 for pS, which may be in error by 0·2, since equilibration of the solutions with accurately known gas mixtures was not very exactly attained. No other measurements appear to have been made at 37·5°.

[1] The equilibrium constants assumed are as follows:

$$\frac{a_{H^+} \times a_{CH_3COO^-}}{C_{CH_3COOH}} = 10^{-4\cdot75},$$

$$\frac{a_{H^+} \times a_{HCO_3^-}}{C_{CO_2}} = 10^{-6\cdot33} \text{ [Hastings and Sendroy, 1925].}$$

$$C_{CO_2} = \frac{0\cdot56}{22\cdot3} \times \frac{P}{760} \text{ [P = partial pressure of } CO_2 \text{ in mm. Hg],}$$

$$a_{CO_3^=} = 10^{-16\cdot55} \frac{C_{CO_2}}{a_{H^+}^2} \text{ [Hastings and Sendroy, 1925].}$$

Activity coefficients for Pb^{++}, CH_3COO^- and HCO_3^- are calculated by the simple Debye-Hückel equation. The equilibrium lead ion concentration is about $\frac{1}{2}$ the ionic strength.

Solubilities of certain lead salts in serum.

We shall now calculate the solubilities in human serum at 37·5° of certain lead salts, the anions of which are present in serum. An approximate value for the solubility product of lead carbonate has just been obtained. For secondary and tertiary lead phosphates, and pyromorphite, we make use of solubility products derived by Jowett and Price [1932] for aqueous solutions at 37·5°.

We take the p_H of serum to be 7·35, its phosphate concentration to be 0·0012 M, and the bicarbonate 0·025 M. The values in Table IV for the solubilities of the lead salts in serum can then be derived[1], in terms of the lead-ion concentrations at equilibrium.

Table IV. *Solubilities of lead salts in serum.*

Salt	Lead-ion concentration	Solubility ratio	P/Pb
$PbCO_3$	$2{\cdot}6.10^{-7}$ M	1000	0
$PbHPO_4$	$1{\cdot}7.10^{-7}$ M	660	1·00
$Pb_3(PO_4)_2$	$1{\cdot}2.10^{-8}$ M	50	0·67
$Pb_5Cl(PO_4)_3$	$2{\cdot}6.10^{-10}$ M	1	0·60

The least soluble salt by far is pyromorphite, $Pb_5Cl(PO_4)_3$ or $PbCl_2.3Pb_3(PO_4)_2$. This is therefore, of these salts, the most likely to be formed when lead salts are added to serum or serum-like solutions, and will be the ultimate reaction product if these salts are the only possible resultants.

In pyromorphite the atomic ratio of phosphorus to lead is 0·60. Brooks found (Table I) a ratio of 0·62 for lead compounds added to a phosphate-chloride mixture, and hence the compound formed was probably pyromorphite, where Brooks believed it to be the tertiary phosphate of lead.

On adding lead chloride to a similar mixture containing also bicarbonate, the present author found a phosphorus-lead ratio of 0·56, by analysis after an hour or two. Probably some lead carbonate was precipitated, the ratio thus being lowered below 0·60.

When Ca^{++} is present, the precipitate contains calcium, and the atomic ratio of calcium to lead is of the order unity. Carbonate may be present in the precipitates, since with Ringer's solution an atomic ratio of 0·52 for P/(Ca + Pb) is found (Table II). The precipitates however probably consist mainly of phosphorus-containing compounds, and we have to decide between compounds of the type $(Pb, Ca)_3(PO_4)_2$ and of the type $(Pb, Ca)_5Cl(PO_4)_3$. The following arguments favour the latter type of formula.

(1) The reaction product in solutions containing no calcium is probably mainly pyromorphite. Pyromorphite is much less soluble than $Pb_3(PO_4)_2$, and

[1] p_{K_2} and p_{K_3} for phosphoric acid are assigned the values 7·165 and 12·18, following Bjerrum and Unmack [1929], and p'_{K_2} for phosphoric acid in serum is assumed to be 6·72 (see later). $\gamma_{HPO_4^=}$ is taken to be 0·235. Hence $a_{HPO_4^=} = 10^{-3\cdot64}$ and $a_{PO_4^{\equiv}} = 10^{-8\cdot47}$, and the calculated figures may be obtained, taking $\gamma_{Pb^{++}}$ to be 0·11, the value used by Jowett and Price [1932] in 0·16 M NaCl.

the entry of calcium into the structures would probably leave the pyromorphite type of solid still the less soluble.

(2) The P/(Ca + Pb) ratio recalculated by the author from the data of Bischoff and Maxwell in serum, 0·56, and the ratio found by these authors in Ringer's solution, 0·52, both favour a substance of ratio 0·60 rather than 0·67.

(3) Pyromorphite occurs in a number of minerals, lead being partly replaced by various metals, including calcium; $Pb_3(PO_4)_2$ is of very limited occurrence in nature. Lead-calcium compounds of the pyromorphite type are therefore known to exist, while those of the tertiary phosphate type are not.

(4) Chloro-apatite, $Ca_5Cl(PO_4)_3$, has also a wide distribution in nature while $Ca_3(PO_4)_2$ has not. On the other hand it seems likely that the calcium compound uniting with lead might be similar to one of those formed in ossification; chloro-apatite may be present in bones [Bassett, 1917] but the small chloride content of bones shows that it is not largely present.

The manometric method.

In most of the reactions which lead compounds are likely to undergo in serum or serum models acid-base changes occur. In systems buffered by CO_2 and HCO_3^-, these changes may be followed by measurements of the pressure of carbon dioxide.

Pressure changes have been followed by the type of manometers and vessels employed by Warburg [1926]. The vessel attached to the manometer has two compartments. Into the small compartment we measure 0·1–0·4 cc. of the lead-containing solution, into the larger one 1–4 cc. of serum or other liquid. The vessel is swept out with a gas-mixture containing some 5 % CO_2 in nitrogen, and is then shaken in a thermostat at 37·5° until the manometer level is steady. The contents of the two compartments are mixed by tilting the vessel to and fro, and pressure readings are then made at intervals, the vessels being shaken between readings.

The reactions of lead ion with serum-models.

A. In absence of calcium. When lead chloride is added to a neutral solution containing chloride, bicarbonate and phosphate, one of the following precipitations would be expected to occur:

(1) $Pb^{++} + HPO_4^{=} \rightarrow PbHPO_4$,
(2) $Pb^{++} + HCO_3^- \rightarrow PbCO_3 + H^+$,
(3) $3Pb^{++} + 2HPO_4^{=} \rightarrow Pb_3(PO_4)_2 + 2H^+$,
(4) $5Pb^{++} + 3HPO_4^{=} + Cl^- \rightarrow Pb_5Cl(PO_4)_2 + 3H^+$.

Owing to the buffering of the phosphate and carbonate-bicarbonate systems additional changes take place, but to a first approximation the H^+ released according to these equations would liberate an equivalent amount of carbon dioxide. Reaction (1) would liberate no gas, and the other reactions 1, 0·67 or 0·60 moles of CO_2 per mole of Pb^{++}.

The results obtained manometrically are indicated in Exps. 1–4 of Table V. It is assumed in the calculations that the evolution of carbon dioxide is due to combination of Pb^{++} with $PO_4^{\equiv}$ according to such equations as (3) or (4), allowance being made for the buffering effects. The ratio of P disappearing

Table V. *Addition of lead chloride to aqueous solutions.*

Exp. No.	Initial concns. (*mM*/l.) of Ca	Phosphate	$NaHCO_3$	p_H	Moles $PbCl_2$ $\times 10^6$	Vols. of solutions	mm.³ CO_2	P/Pb
1	0	1·5	24·6	7·50	2·4	0·24, 4·0	36·9	0·68
2	0	1·5	26·1	7·31	3·0	0·2, 3·5	44·5	0·59
3	0	0·75	16·4	7·32	3·0	0·3, 4·0	48·0	0·62
4	0	1·5	24·6	7·50	4·0	0·4, 4·0	57·4	0·62
5	1·25	1·46	24·0	7·49	1·2	0·12, 4·0	35·6*	1·26*
6	1·25	1·46	24·0	7·49	1·2	0·12, 4·0	33·8	1·20
7	1·25	1·46	24·0	7·49	2·5	0·25, 4·0	55·5	0·95
8	2·5	1·42	23·4	7·48	2·5	0·25, 4·0	77·6	1·29
9	5·0	1·35	22·2	7·45	2·45	0·245, 4·0	116·0	1·90
10	1·12	1·47	25·7	7·30	3·0	0·20, 3·58	63·9	0·83
11	1·66	1·45	25·4	7·29	3·0	0·20, 3·62	67·2	0·86
12	3·21	1·40	24·6	7·28	3·0	0·20, 3·74	86·6	1·10

NaCl was also present, bringing the ionic strength to 160 *mM*/l.

* In Exps. 5–9 the values given are those obtained 5 hours after the addition, in Exps. 10–12 1 hour after.

to Pb added is found to be about 0·62, the same value as that of Brooks. Both values may be somewhat in error (the manometric value by perhaps 3–5 %) but they indicate that the product formed is probably mainly pyromorphite. The agreement of the two values rules out much formation of $PbCO_3$, the probable formation of which in experiments referred to earlier may be due to conditions less favourable to attainment of equilibrium.

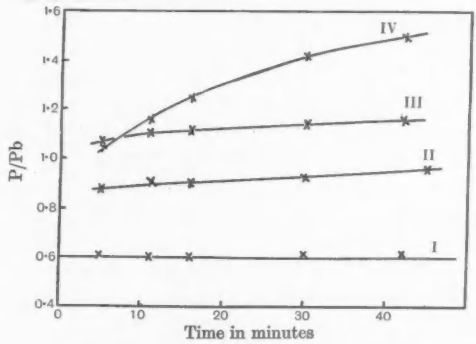


Fig. 1. Addition of lead chloride to Ringer's solutions.

The evolution of gas after addition of lead in terms of the P/Pb ratio is shown by Curve I, Fig. 1 (Exp. 4), and by Curve I, Fig. 2 (Exp. 2). It is complete in about 5 minutes, and takes place at about the same rate as when HCl is added to Ringer's solution. The precipitation must therefore be rapid—a

turbidity is visible immediately—the lag in evolution of gas being due to the limited surface and rate of shaking of the vessels.

B. In presence of calcium. When lead chloride is added to solutions of the Ringer type containing calcium, the reactions are more complicated. The results (Table V, Exps. 5–12) have been calculated as before.

The evolution of gas is rapid for about 5 minutes and continues at a diminishing rate for some hours. Curves II and III of Fig. 2 show the evolution

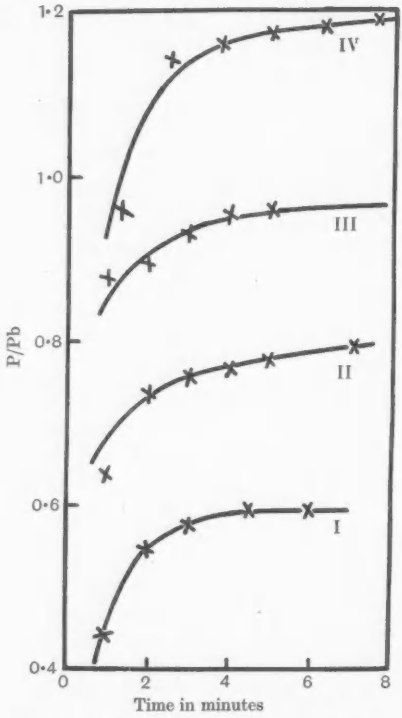


Fig. 2. Addition of lead chloride to Ringer's solution or serum.

of gas in Exps. 11 and 12, corrected for the lag of evolution on the assumption that the lag is the same as in similarly conducted experiments in absence of calcium, the chemical reaction being taken to be instantaneous in the latter case. The curves represent the extent to which Pb^{++} has reacted with $PO_4^{\equiv}$ at various times. After a very short time the P/Pb ratio exceeds 0·60, and is higher for the system in which the initial calcium ion concentration is greater. In Fig. 1 are shown gas evolutions, expressed in terms of P/Pb ratios, over greater time intervals: Curves II, III and IV illustrate Exps. 7, 8 and 9. The initial $[Ca^{++}]$ rises from Exp. 7 to 9, and so does the P/Pb ratio.

In Table VI the data are arranged to show that the initial Ca^{++}/Pb^{++} ratio in the solution, before precipitation has begun, determines largely the value of P/Pb that is reached after some time.

Table VI.

Exp. No.	P/Pb	Initial Ca^{++}/Pb^{++}	Initial Ca^{++}	$0{\cdot}60+0{\cdot}155$ Ca^{++}/Pb^{++}
10	0·83	1·3	1·12	0·80
11	0·86	2	1·66	0·91
7	0·95	2	1·25	0·91
12	1·10	4	3·21	1·22
6	1·20	4·2	1·25	1·25
5	1·26	4·2	1·25	1·25
8	1·29	4	2·50	1·22
9	1·90	8·2	5·00	1·88

The findings may be interpreted as follows. As in the absence of calcium, pyromorphite, $Pb_5Cl(PO_4)_3$, precipitates rapidly. Calcium is however able to compete with lead for a place in the molecular structure, and the actual substance produced is $(Pb, Ca)_5Cl(PO_4)_3$. The extent to which calcium can enter the precipitate by competition with lead depends on the Ca/Pb ratio in the solution. This ratio rises as precipitation proceeds, but the initial value serves as a measure of the effect. The ratio of phosphorus disappeared to lead added therefore reaches a value 0·60 or higher very rapidly: the extent to which the value is higher than 0·60 depends on the Ca^{++}/Pb^{++} ratio, and is roughly proportional to it, as shown in the last column of Table VI. After the initial rapid precipitation of calcium and lead, the removal of phosphate from the solution continues, but at a much slower rate. Aggregation of the precipitate may make it more difficult for Ca^{++} to react with it: insolubility of the precipitate may tend to prevent replacement of Pb by Ca, and there must be a limit to the extent to which Ca can add on to the precipitate to form a layer containing little or no Pb.

The above view of the phenomena is not completely satisfactory. It does not explain why the reaction continues so much more rapidly in Exp. 9 (Curve IV, Fig. 1) than in the others.

An alternative explanation of the facts can be proposed. Calcium tends to add on to the precipitated $Pb_5Cl(PO_4)_3$ until it is converted into $Pb_5Cl(PO_4)_3 . Ca_5Cl(PO_4)_3$. In support of this, the P/Pb ratio reaches values near 1·20 in four experiments.

The single very high value for P/Pb can be explained by supposing that in Exp. 9, where the $[Ca^{++}]$ is the highest of the series, precipitation of $Ca_3(PO_4)_2$ takes place. All the solutions are supersaturated with respect to $Ca_3(PO_4)_2$, that in Exp. 9 being most highly supersaturated. In Exp. 9 some 90 % of the phosphate is removed from solution, and hence shortage of phosphate may be the limiting factor to reaction.

We have hardly sufficient data to decide between the two possibilities raised: (1) that the Ca/Pb ratio in the precipitate can vary continuously, and

(2) that the Ca/Pb ratio tends to rise to unity and stop there, unless calcium phosphate should precipitate as well.

The second explanation appears rather more probable, taking into account also the results obtained with serum.

Calculation of results.

The details of the calculation for Exp. 4 are given below. The terminology of Warburg [1924; 1925] is employed. Pressures are expressed in such a unit that 10,000 mm. equal 1 atmosphere, and concentrations often in mm.[3] per cc., where 22,300,000 mm.[3] equal 1 mole[1].

An hour after adding 0·4 cc. of 0·01 *M* $PbCl_2$ to 4·0 cc. of solution, the increase in pressure was 50·2 mm., corresponding to an evolution of 57·4 mm.[3] of CO_2 in the system:

$$\Delta h = \Delta p_{CO_2} = +\,50{\cdot}2. \quad k^{\text{Ring.}}_{CO_2} = 1{\cdot}144, \; x_{CO_2} = 57{\cdot}4 \text{ (mm.}^3\text{)}.$$

The initial pressure of CO_2 was 420 mm. (approximately, from the also approximate composition of the gas-mixture), and the final pressure is therefore 470·2: $p_{CO_2} = 420$, $p'_{CO_2} = 470{\cdot}2$.

The initial $[HCO_3^-]$ (0·0246 *M*) is given by:

$$x_{HCO_3^-} = 0{\cdot}0246 \times 1{\cdot}10^{-3} \times 2{\cdot}23\,.\,10^7 = 550 \text{ (mm.}^3\text{/cc.)}.$$

When both dilution and evolution of CO_2 are taken into account, the final $[HCO_3^-]$ concentration is 487:

$$x'_{HCO_3^-} = \frac{550 \times 4 - 57{\cdot}4}{4{\cdot}4}.$$

The p_H values are now calculated by means of the equation[2]

$$p_H = 6{\cdot}13 - \log \frac{0{\cdot}056\, p_{CO_2}}{x_{HCO_3^-}},$$

which yields the values 7·499 and 7·397. The p_H thus decreases during the reaction by about 0·102.

The ratio of $HPO_4^=$ to $H_2PO_4^-$ in the solution is altered by the change of p_H. The amounts of the ions are decreased by the precipitation, and to calculate a trial result we assume that the moles of phosphate disappearing are 0·60 times the g.-atoms of lead added. The phosphate initially in solution in the vessel is $6{\cdot}10^{-6}$ moles, and finally (on our assumption) $3{\cdot}6\,.\,10^{-6}$.

We calculate the moles of $H_2PO_4^-$ and $HPO_4^=$ initially and finally present by means of the relation[3]

$$p_H = 6{\cdot}72 + \log \frac{C_{HPO_4^=}}{C_{H_2PO_4^-}},$$

[1] Warburg uses the value 22,400,000, but according to E. J. Warburg [1921] the lower value is more accurate for CO_2.

[2] The value 6·13 is that used by Hastings, Murray and Sendroy [1926–7].

[3] The data of Bjerrum and Unmack [1929] yield the value 6·726 for solutions of ionic strength 0·16, which however do not consist mainly of sodium chloride. Using the p_{K_2} value of these authors, namely 7·165, and taking a rather inaccurate value 0·458 for $\Delta p_{K'_2}$ for isotonic salt solutions from the data of Jowett and Millet [1929], we derive a value 6·707. Sendroy and Hastings [1926–7] use the value 6·66 for serum, but the value 6·72 is preferred here for serum also.

and obtain the values $8{\cdot}56.10^{-7}$ and $6{\cdot}26.10^{-7}$ for $H_2PO_4^-$, and $51{\cdot}44.10^{-7}$ and $29{\cdot}74.10^{-7}$ moles for $HPO_4^=$. Hence $2{\cdot}3.10^{-7}$ moles of $H_2PO_4^-$ disappear and $21{\cdot}7.10^{-7}$ of $HPO_4^=$. Each mole of $H_2PO_4^-$ reacting with lead releases 2 moles of CO_2, and each mole of $HPO_4^=$ 1 mole of CO_2.

The CO_2 appearing in the system should therefore be given by

$$= 2{\cdot}23.10^{-7} \times (21{\cdot}7.10^{-7} + 2 \times 2{\cdot}3.10^{-7}) = 58{\cdot}7.$$

A correction must be applied to this value, since a small amount of $CO_3^=$ is present in the solution, and by the dilution and acidification some of this is transformed to HCO_3^- with absorption of CO_2.

The concentration of $CO_3^=$ is given by[1]

$$x_{CO_3^=} = \frac{(x_{HCO_3^-})^2}{227\, p_{CO_2}}.$$

Hence $x_{CO_3^=}$ is initially 3·17, and finally 2·22; the amount of $CO_3^=$ (and CO_2) disappearing from the system is therefore 2·9 mm.[3]

Hence the calculated formation of CO_2 in the system is 55·8, while the observed value is 57·4. The observed value for P/Pb is therefore not 0·60, but 0·62.

The other experiments have been calculated similarly; the trial value for P/Pb must be near the observed.

The reaction of lead ion with serum.

When lead chloride is added to cow-serum, the results are very similar to those which follow its addition to Ringer's solution containing calcium. The evolution of gas becomes slow after 5 minutes[2], but continues for perhaps an hour. The rate of the slow process is of the same order as in Ringer's solution, although the particles formed precipitate in Ringer's solution after a few minutes, and do not in serum. The favouring influence of small particle size in serum may be offset by protein adsorption hindering reaction.

The buffering of serum results in formation of CO_2 from only about a half of the H^+ developed in the system. The calculation of the acid formed (x_s) has been made by the methods of Warburg [1925], using the constants for carbonic acid of Hastings, Sendroy and Van Slyke [1928]. The ratio of $H_2PO_4^-$ to $HPO_4^=$ reacting with lead is determined by the final p_H of the system. As before, the data have been interpreted on the assumption that Pb^{++} reacts with $PO_4^{\equiv}$, and the ratios of P/Pb derived from the gas evolved after 60–90 minutes are shown in Table VII. Three different samples of serum were used, the last one being kept in a refrigerator for 3 days before use.

The observed values of P/Pb may be in error by some 10 %, and the variations shown in Table VII may not be real. The mean observed value is

[1] The value used for the equilibrium constant has been interpolated from the data of Walker, Bray and Johnston [1927]; it is 102 in these authors' units, 227 in ours.

[2] Shown in Curve IV, Fig. 2, which illustrates Exp. 3*a*; the rate of evolution is corrected to that due to chemical reaction by taking lag due to physical causes to be the same as when HC is added to serum.

1·19. By direct analysis Brooks found a value 1·1, and Bischoff and Maxwell 1·29. As the manometric value lies between these, we have evidence that the main process actually occurring is disappearance of $PO_4^{\equiv}$, and the value is in agreement with the view that the product of reaction is the compound $Pb_5Cl(PO_4)_3.Ca_5Cl(PO_4)_3$.

Table VII. *Addition of lead chloride to cow-serum.*

Exp. No.	Moles $PbCl_2$ $\times 10^6$	Vol. of solutions	x_s	Final p_H	P/Pb
1*a*	2·5	0·25, 3·0	74·6	7·04	1·01
1*b*	2·5	0·25, 3·0	79·3	7·04	1·07
2*a*	2·93	0·195, 3·5	94·4	7·17	1·15
2*b*	3·0	0·20, 3·5	102·2	7·17	1·21
3*a*	3·0	0·20, 3·5	115·0	7·19	1·37
3*b*	3·0	0·20, 3·5	110·9	7·19	1·32

The calcium contents of the sera were not estimated. The phosphate concentration in one case was 0·0023 *M*. Both calcium and phosphate concentrations were probably similar to those in the aqueous solutions in which P/Pb reached values near 1·2, and our experiments do not show evidence of any differences between the reaction in Ringer's solution and serum, except that in serum there are no values which can be attributed to formation of calcium phosphate. This agrees with the fact that injections of lead preparations do not cause cardiac symptoms similar to those produced by injections of colloidal calcium phosphate.

The reactions of other lead compounds.

Experiments were made with the colloidal preparation S_7, described by Brooks [1927], and with similar preparations containing no calcium. The colloids, which contain mainly metallic lead and lead hydroxide, were treated with oxygen to oxidise the lead, centrifuged slightly, and added (as before) to phosphate-containing solutions in absence or presence of calcium chloride, or to serum.

Some carbon dioxide from the gas mixture was however absorbed by the alkaline colloids before they were mixed with the solutions (although the colloid was covered with liquid paraffin) and as the absorption was variable the manometric data can only be interpreted semi-quantitatively. As far as they go, they show that the products of reaction are the same as when lead chloride reacts.

In the absence of calcium the manometric change is about 90 % complete in 5 minutes: the proportion of lead that has reacted will be less than this, since lead carbonate in reacting brings about less gas exchange than does lead hydroxide, which is likely to react more rapidly. The reaction when calcium is present is also rapid. The rapidity of the reactions must be due to the small size of the particles. It is intelligible that calcium should react well with the particles: the $[Pb^{++}]$ at the reacting surface has as an upper limit the

solubility of lead hydroxide (about $0{\cdot}0005\,M$), while the $[Ca^{++}]$ in the solution is $0{\cdot}0025\,M$.

Lead carbonate when finely divided—prepared by adding oxidised S_7 to a solution containing bicarbonate—also reacts rapidly with phosphate, the reaction being about half complete in 5 minutes. Brooks found the reaction to be slow when a coarse suspension was used.

The slow completion of the reaction of S_7 with phosphate, which Brooks attributed to slow reaction of lead carbonate with phosphate, is probably due to a slow completion of the reaction of calcium.

Experiments have been made with two further preparations of lead, (1) a colloidal solution of the lead salt of benzenesulphonylglycine[1], and (2) a soluble compound[2] known as R 232, in solutions of which a very low concentration of Pb^{++} is maintained in equilibrium with a lead compound. These compounds react with phosphate in absence and presence of calcium in a very similar manner to lead chloride, the rates of reaction being similar.

Summing up, we may say that Pb^{++} reacts almost instantaneously with phosphate, and so do fairly soluble lead salts (lead hydroxide and lead benzenesulphonylaminoacetate). The less soluble lead carbonate reacts rapidly if finely dispersed, and the very insoluble lead sulphide reacts very slowly [Brooks, 1928]. The reactivity of lead compounds evidently depends on the rate at which they can form Pb^{++}, solubility and surface both being of importance. No other factors have yet manifested themselves.

Lead ion concentrations during the reactions.

Lead ion concentrations have been measured after addition of lead compounds to solutions of the Ringer type. Measurements were made by the technique of Millet [1929], standardisation in terms of $[Pb^{++}]$ being effected by use of a standard solution[3].

1 cc. or less of S_7 or dilute lead chloride is added to some 25 cc. of solution (containing $0{\cdot}025\,M$ $NaHCO_3$, and of p_H 7·55–7·6), and after the solutions are mixed E.M.F. measurements are begun at once. The solutions mixed are indicated in Table VIII, and the Pb^{++} concentrations during the first half-hour are shown in Fig 3.

Precipitation of Pb^{++} progresses so rapidly that before measurements are begun the Pb^{++} concentrations have fallen in every case below $1{\cdot}10^{-7}\,M$: they continue to fall, but at a decreasing rate.

[1] This preparation, which contained gum acacia and sodium thiosulphate, was made by Prof. I. M. Heilbron and Dr B. Beilensohn in the Department of Organic Chemistry, University of Liverpool.

[2] Supplied to Prof. W. Blair Bell by Dr W. Collier, of the Koch Institute, Berlin.

[3] We assume here for simplicity that all the lead is present as lead ion. Probably association of Pb^{++} to $PbCl^+$ takes place, but its extent will be fairly constant in the solutions concerned. The lead amalgam electrode apparently functions correctly at these low Pb^{++} concentrations, owing to its regulation by a dispersed lead salt, and in the case of S_7 to removal of oxygen by metallic lead.

Table VIII. *Addition of lead compounds to various solutions.*

Curve	Compound added	mM Phosphate	mM Ca	Lowest lead ion concentrations observed: pC_{Pb}^{++}	Time (mins.)	Compound being formed
VI	S_7	0	0	7·32	27	$PbCO_3$
V	$PbCl_2$	1	1	8·59	72	$Pb_5Cl(PO_4)_3$
	$PbCl_2$	1	1	9·00	280	
IV	S_7	1·5	1	8·96	42	$Ca_5Cl(PO_4)_3$
III	S_7	1·5	1	9·46	170	
II	S_7	1·5	0	9·54	66	$Pb_5Cl(PO_4)_3$
I	S_7	1·5	0	9·57	30	

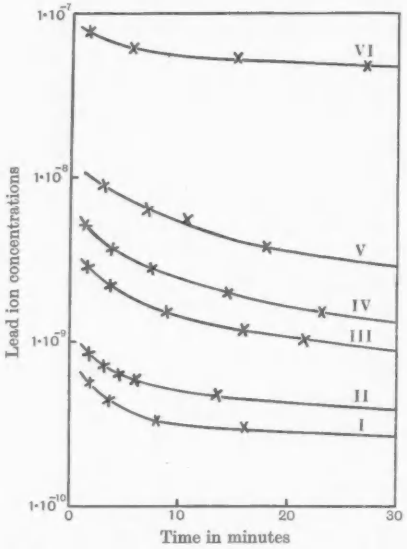


Fig. 3. Lead ion concentrations during the precipitations.

The experimental curves are widely spread out in Fig. 3, indicating that different reactions are occurring, since the $[Pb^{++}]$ must tend towards the solubility of the compound which has been formed.

The experiments were made at 25°, and our solubility data relate to 37·5°. The calculated equilibrium Pb^{++} concentrations (at 37·5°) for our experimental conditions are for $PbCO_3$ $10^{-6\cdot8}$, for $Pb_3(PO_4)_2$ $10^{-8\cdot1}$, and for $Pb_5Cl(PO_4)_3$ $10^{-9\cdot8}$. These figures are fortunately widely different.

When no phosphate is present we should expect formation of $PbCO_3$, and this apparently takes place, for Curve VI lies at a Pb^{++} level of about 10^{-7}.

In no case is $Pb_3(PO_4)_2$ the final product of reaction, unless the temperature coefficient of the solubility of this salt is extremely high. Curves I–V are tending towards values below $10^{-9\cdot5}$, and the reaction product is therefore

either $Pb_5Cl(PO_4)_3$ or a compound of similar solubility. This deduction is made from E.M.F. measurements alone, and is an independent confirmation of the conclusion already reached.

The slope of the curves is greater when calcium is present (III–V) than when it is absent (I, II). It might therefore be inferred from this alone that calcium takes some part in the reaction. With our previous knowledge, we may note in the curves a confirmation of the view already advanced that calcium and lead ions compete for places in the solid phase. So far we have regarded the effects on the calcium; now we see that the lead ion is affected, in that its concentration does not reach a low level as rapidly in the presence of calcium as in its absence.

The data do not tell us whether the lead-calcium compound is more or less soluble in our solutions than pyromorphite, since measurements were not pursued to attainment of equilibrium. On theoretical grounds, if the lead calcium compound is the stable one in presence of calcium, it must also be the less soluble in terms of lead ion, but need not be much less soluble.

Summary.

The reactions of lead compounds, and in particular Pb^{++}, with serum and serum-models have been studied by a manometric method, with the lead electrode and with the help of solubility data. The manometric method is not specific, but is valuable for measuring rates of reaction.

When $CO_3^{=}$ is the only precipitant ion, lead carbonate is formed. In the presence of phosphate (and chloride) the product is pyromorphite, $Pb_5Cl(PO_4)_3$. When calcium also is present, as in serum, the product approximates to $Pb_5Cl(PO_4)_3.Ca_5Cl(PO_4)_3$, an equimolecular compound of pyromorphite and chloro-apatite. Lead ion reacts very rapidly, but the union of calcium is less rapid than that of lead and may be incomplete under unfavourable conditions. Solubility and extent of surface determine the rate of reaction of solid lead compounds with phosphate.

An approximate value is obtained for the solubility product of lead carbonate at 37·5°. The solubilities in serum of $PbCO_3$, $PbHPO_4$, $Pb_3(PO_4)_2$ and $Pb_5Cl(PO_4)_3$ are calculated, pyromorphite being the least soluble of the four salts.

REFERENCES.

Aub, Reznikoff and Smith (1924). *J. Exp. Med.* **40**, 151.
—— and Reznikoff (1924). *J. Exp. Med.* **40**, 189.
Bassett (1917). *J. Chem. Soc.* **111**, 620.
Bischoff and Maxwell (1928). *J. Biol. Chem.* **79**, 5.
Bjerrum and Unmack (1929). *Det. Kgl. Danske Videnskabernes. Selskab. Mathematisk-fysiske Meddelester.* **9**, 1.
Brooks (1927). *Biochem. J.* **21**, 766.
—— (1928). *J. Phys. Chem.* **32**, 1717.
Hastings, Murray and Sendroy (1926–7). *J. Biol. Chem.* **71**, 723.
—— and Sendroy (1925). *J. Biol. Chem.* **65**, 445.
—— —— and Van Slyke (1928). *J. Biol. Chem.* **79**, 183.
Jowett and Millet (1929). *J. Amer. Chem. Soc.* **51**, 1004.
—— and Price (1932). *Trans. Faraday Soc.* **28**, 668.
Millet (1929). *Trans. Faraday Soc.* **25**, 147.
Scremin (1925). *Arch. exp. Path. Pharm.* **105**, 49.
Sendroy and Hastings (1926–7). *J. Biol. Chem.* **71**, 783.
Walker, Bray and Johnston (1927). *J. Amer. Chem. Soc.* **49**, 1235.
Warburg (1924). *Biochem. Z.* **152**, 51.
—— (1925). *Biochem. Z.* **164**, 481.
—— (1926). Über den Stoffwechsel der Tumoren, Berlin, 1–7, 159.
Warburg, E. J. (1921). *Biochem. J.* **15**, 1.

CCLI. SOME EXTRACTS OF PARATHYROID GLANDS CONTAINING AN ANTI-GROWTH FACTOR. I.

By CYRIL JACK EASTLAND, NORMAN EVERS and JAMES HENRY THOMPSON.

From the Laboratories of King's College, London, The Royal College of Surgeons, and Allen and Hanburys.

(*Received November 4th, 1932.*)

Previous work on the parathyroid gland [Collip, 1925; Collip and Clark, 1925; Hjort, Robison and Tendick, 1925] has shown the presence of a serum-calcium raising factor. From experiments on animals injected with a preparation of this gland one of us [Thompson, 1931] was led to suspect the presence of an anti-growth factor. It seemed possible that this activity might be due to the serum-calcium raising factor itself or to the presence of a specific anti-growth factor as an impurity in the extract. A series of extracts was therefore prepared with the intention of eliminating the Collip factor as far as possible. Collip and Clark [1925] have shown that the latter is precipitated at p_H 4·8. All extracts, however prepared, were adjusted to this p_H and allowed to stand until no further precipitation took place.

The preliminary extracts were prepared by the following methods.

(1) Aqueous extraction.

(2) Extraction with 95 % alcohol.

(3) Extraction with 80 % alcohol containing 5 % by weight of sulphuric acid, afterwards neutralising with sodium hydroxide to p_H 4·8, filtering off the sodium sulphate and concentrating *in vacuo*.

(4) Collip's method, heating with dilute hydrochloric acid, making alkaline to p_H 8 to 9, precipitating inert matter by bringing to p_H 5·6, acidifying to p_H 4·8 and allowing to precipitate.

(5) Method (4), but previously defatting the glands with acetone.

Acid alcohol extraction.

The results of animal tests with these fractions encouraged us to adopt method (3) as a basis for further investigations. Berman [1926] used a similar method for preparing an extract containing the Collip factor, but the acid alcohol extract was neutralised to litmus, thus retaining the Collip factor in solution. Incidentally it appears that Berman's extracts must have contained sodium sulphate, the alcohol concentration not being sufficiently high to remove it.

Method (3) was therefore elaborated for use with larger quantities of material. 100 g. of fresh parathyroid glands, freed from adherent fat and connective tissue and finely minced, were shaken continuously for 48 hours with 1 litre of alcohol (85 % by volume) containing 5 % by volume of sulphuric acid. The mixture of acid and alcohol must be made slowly and the temperature kept low, otherwise sodium ethyl sulphate is formed which appears in the final product. This compound has been found to have no growth-retarding action on rats.

The gland residue was then strained off, and the liquid was neutralised to methyl red by the gradual addition of 50 % sodium hydroxide solution, keeping the mixture cool. Alcohol was then added so as to give a final concentration of 75 % by volume in the mixture. This ensures the almost complete precipitation of the sodium sulphate, on cooling to about 5°. After keeping at this temperature overnight the sodium sulphate and other precipitated material were removed by filtration through a Büchner funnel. The filtrate was concentrated under reduced pressure at about 15–20 mm. until all the alcohol was removed. The volume of the concentrated liquid was adjusted so that 1 cc. was equivalent to 2 g. of parathyroid gland, and the p_H was adjusted

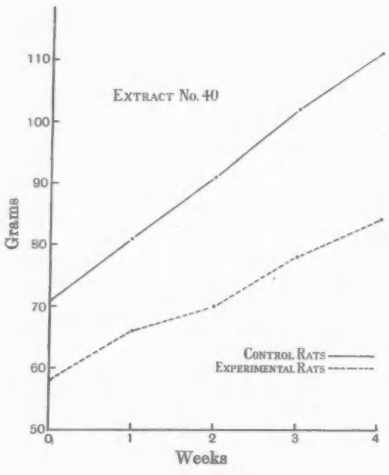


Fig. 1.

to 4·8. The extract was then allowed to stand at a temperature of about 5° until no further precipitation occurred, when it was filtered through a Seitz filter. The extract was used in this condition for animal tests without the addition of a preservative. For a description of the method of testing and for the physiological evidence for the anti-growth factor see Robinson and Thompson [1932].

A number of extracts prepared by this method have given definite evidence of the presence of an anti-growth factor. Fig. 1 shows the effect of such an extract on the growth of rats. Six rats were used as controls and six were given daily injections of 2 cc. of the extract, other conditions being the same.

Defatting by acetone. The large amount of fat in parathyroid glands prevents the efficient penetration of the gland material by the cold acid alcohol. Previous treatment of the minced glands with cold acetone appeared to give a more complete extraction. The fat so obtained appeared to possess a slight activity, as the following experiment shows.

200 g. of the fat extracted by acetone were dissolved in 750 cc. of light petroleum and shaken for 24 hours with 1 litre of 70 % by volume alcohol containing 10 % by volume of sulphuric acid. After separation the light petroleum layer was washed with 70 % alcohol and the combined acid alcohol extracts were treated as described above. The final concentration of the solution was, however, adjusted so that 1 cc. was equivalent to approximately 25 g. of gland instead of 2 g. The effect of this extract on rat-growth is shown in Fig. 2. The difference in the average increases in weight over 4 weeks is insignificant. Nevertheless if the last two weeks only be considered the growth of the injected rats was practically stationary during this period while the average increase of the controls was 13 g.

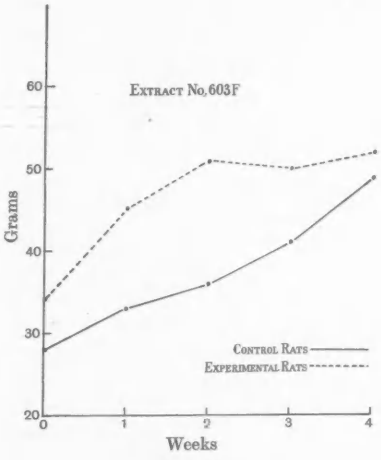


Fig. 2.

Defatting with benzene. In order to avoid this loss of active material benzene was added to the minced gland with the acid alcohol and the mixture was shaken together. This was an improvement on previous acetone extraction in removing the fat and allowing better extraction of the gland material.

400 g. of parathyroid glands were minced and shaken for 48 hours with

700 cc. of benzene and a cooled mixture of 300 cc. of sulphuric acid, 1800 cc. of water and 900 cc. of 95 % alcohol. The acid alcohol was separated and treated as in the first method.

Removal of fat by heat. The minced glands were mixed with alcohol (30 % by vol.) containing 5 % by vol. of sulphuric acid and heated to 70° for a few minutes until the fat was completely melted and rose to the surface. After cooling, the cake of fat was removed and the acid alcohol and gland material were shaken together for 48 hours. The liquid was filtered, neutralised with sodium hydroxide solution, and alcohol added to 75 % by vol. concentration as in the first method. The extract obtained was found to be active (see Fig. 3, extract No. 601). The fat obtained in this method was dissolved in benzene and extracted with acid alcohol. The results confirmed the previous finding that the fat contains some of the anti-growth factor.

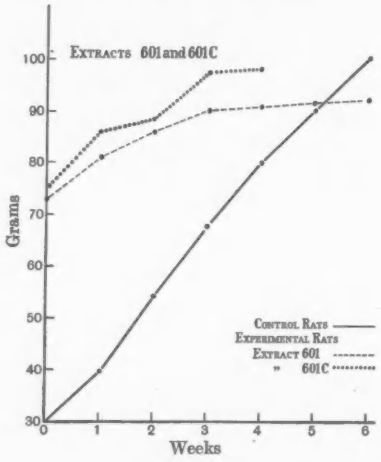


Fig. 3.

Salting-out. Extracts prepared by the above methods have been salted out with sodium sulphate at p_H 4·8 with a view to removing the Collip factor more completely. This has been repeated a number of times and in all cases in which it has been done an increase in the anti-growth potency has been observed. The usual procedure was to saturate the solution at p_H 4·8 with sodium sulphate at room temperature and filter off the precipitate formed, which was usually small in amount. The precipitate was redissolved in a smaller volume of water at p_H 4·8 and again salted out in the same way. The combined filtrates were treated with alcohol to a concentration of 75 % by volume and the sodium sulphate was filtered off after crystallising at 5° overnight. The alcohol was removed from the filtrate under reduced pressure and the concentrate was made up to the original volume with water.

The results on growth of such an extract (40 SO) are shown in Fig. 4.

The total average increase in the injected rats is only half the increase obtained with the extract before salting-out (40). Table I. Tests on a solution of the salted-out precipitate have failed to show any activity.

Alkaline extraction. 1200 g. of minced parathyroid gland were heated to 40–45° with 2400 cc. of 2 % sodium hydroxide solution and kept at that temperature for a few minutes, then at 37° for 24 hours, toluene being added as a preservative. After separation of the fat the liquid was neutralised to p_H 7 and centrifuged. The clear liquid was treated with alcohol until the concentration was 75 % by volume, the sodium sulphate was removed in the usual way, and the filtrate concentrated as usual. This extract appeared to have little or no activity.

Treatment with activated charcoal. 300 cc. of an active extract (601) were shaken with 6 g. of activated charcoal (Darco) for half an hour and filtered. The filtrate (601 C) still contained the anti-growth factor in undiminished amount (Fig. 3).

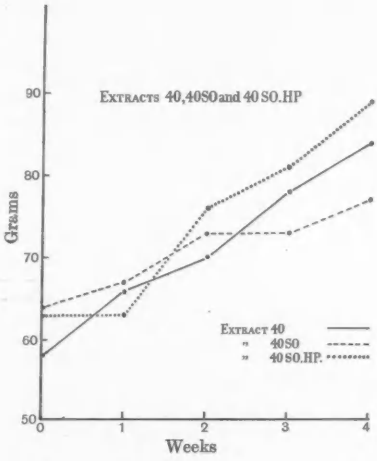


Fig. 4.

Treatment with hydrogen peroxide. 1 cc. of 30 % hydrogen peroxide was added to 300 cc. of an active extract (40 SO) and the mixture kept overnight at 22°. The excess of hydrogen peroxide was removed by the addition of manganese dioxide which was filtered off when the hydrogen peroxide was completely decomposed. This treatment appears in some cases to destroy the whole of the anti-growth activity [Robinson and Thompson, 1932] but the destruction is not always complete; for example, treatment with hydrogen peroxide reduced the potency of extract (40 SO) and the extract so obtained (40 SO.HP) appeared to have about the same potency as the original extract (40) before the salting-out process had increased its activity (see Fig. 4).

Chemical properties of the extracts. Extracts prepared by the acid alcohol method contain about 3 % of total solids, of which about 1·5 % are ash. The nitrogen content is about 0·2 %. Picric acid gives a slight precipitate, and phosphotungstic acid a heavy one. They give marked xanthoproteic and Millon's reactions which are reduced in intensity by the salting-out treatment and by treatment with activated charcoal. Ehrlich's reaction for tryptophan is also positive, but is very faint when applied to the extract after treatment with hydrogen peroxide. The extract of the fat was very low in solids and gave none of these reactions.

Summary.

1. Extracts have been prepared from parathyroid glands containing a factor which retards the normal growth of rats.

2. Extraction with acid alcohol gives active extracts.

3. Alkaline extraction gives little or no activity.

4. Removal of the fat gives better extraction, but the fat may contain some of the activity.

5. Salting-out increases the potency of active extracts, the salted-out material having no activity.

6. Treatment with activated charcoal does not affect the potency.

7. Treatment with hydrogen peroxide may partially or completely destroy the activity.

REFERENCES.

Berman (1926). *Amer. J. Physiol.* **75**, 358–65.
Collip (1925). *J. Biol. Chem.* **63**, 395.
—— and Clark (1925). *J. Biol. Chem.* **66**, 133.
Hjort, Robison and Tendick (1925). *J. Biol. Chem.* **65**, 117.
Robinson and Thompson (1932). *J. Physiol.* **76**, 303.
Thompson (1931). *J. Physiol.* **71**, xvii.

CCLII. THE COLORIMETRIC DETERMINATION OF ADRENALINE IN SUPRARENAL GLAND EXTRACTS.

By JAMES HARALD BARKER, CYRIL JACK EASTLAND and NORMAN EVERS.

From the Laboratories of Allen and Hanburys.

(*Received November 4th, 1932.*)

Soon after the commencement of the manufacture of extract of suprarenal cortex for the treatment of Addison's disease it became evident that even moderate traces of adrenaline could not be tolerated owing to the large doses sometimes employed, and it became necessary to find a colorimetric method for the rapid estimation of adrenaline at certain stages of the process.

Various tests were tried on these comparatively crude extracts and the ammonium molybdate test, described later, was adopted provisionally since it was found that extracts which showed less than 1 part in 10,000 of adrenaline by this test, when injected, did not give rise to any of the physiological reactions characteristic of adrenaline.

With improved methods of manufacture much purer extracts were obtained which proved to contain no adrenaline when estimated biologically. Nevertheless, such extracts when tested colorimetrically with ammonium molybdate always gave some colour, usually equivalent to about 0·5 part per 10,000 of adrenaline but occasionally as high as 2 parts per 10,000. It was obvious, therefore, that it was useless to depend upon this test and it was decided to investigate further the subject of colorimetric determination of adrenaline in order to find a test which could be relied upon when applied to such gland extracts.

The numerous colour tests for adrenaline described in the literature have been grouped under three headings for the purposes of this investigation:

(*a*) those depending primarily on the formation of a red oxidation product;

(*b*) those depending on the presence of the catechol grouping;

(*c*) miscellaneous.

Included under heading (*a*) are the following:

(1) ferric chloride reaction, (2) potassium iodate reaction and its modifications, (3) potassium persulphate reaction, (4) mercuric chloride reactions, (5) other oxidising agents, including hydrogen peroxide, potassium ferricyanide, permanganate and dichromate, sodium bismuthate, sodium hypobromite, manganese dioxide and the halogens.

Under (*b*) we have included the ammonium molybdate test and Folin's test.

Miscellaneous tests include diazobenzenesulphonic acid, Azzolini's test [1931] and the ninhydrin test [Orru, 1929]. Another reaction (depending upon the formation of a blue ferrous-adrenaline complex) consists in the addition of a trace of ferrous sulphate followed by sufficient sodium phosphate (Na_2HPO_4, $12H_2O$) to bring the mixture to neutrality. A blue colour appears which is fairly stable but which rapidly fades through a purple colour if too much alkali is added. The reaction did not seem sensitive enough for the estimation of traces of adrenaline.

A preliminary examination of any test which appeared suitable for quantitative work was made and it was soon found that some of the tests described were extremely unreliable even when considered as qualitative tests, *e.g.* those described by Azzolini [1931], Paget [1931] and Orru [1929], this last being non-specific.

The reaction with mercuric chloride, originally suggested by Comessatti [1909] and modified by Ewins [1910], was not found to be very sensitive, although its application to gland extracts has been reported by Paget and Lohéac [1928] and Bailly [1924]. The modification of Comessatti's reaction described by Stuber, Russmann and Proebsting [1923] consists in the addition of a sulphanilic acid, potassium iodate and mercuric chloride reagent to adrenaline solution and boiling for 1 minute. They claim an extraordinary sensitivity of 1 in 100 millions for this test. Viale [1930] has applied this test to blood and confirms some of Russmann's statements. This test was, however, found to be unreliable, since the reagents themselves when mixed, on boiling produced an orange-brown colour which rapidly deepened on standing, due to the liberation of iodine. Purification of the sulphanilic acid produced no improvement and attempts to remove the excess of iodine with sodium thiosulphate were unsuccessful owing to the liberation of sulphur.

The reaction between adrenaline and diazotised sulphanilic acid described by Friend [1923] for quantitative application to urine (using a colorimeter) was also found unsatisfactory. No definite red colour was obtained either with pure adrenaline or with urine to which adrenaline had been added. Moreover, the orange-yellow colour which was obtained, while certainly somewhat stronger in the presence of adrenaline, was also given by a blank determination, making the colorimetric estimation of traces of adrenaline unreliable, if not impossible. The sulphanilic acid was recrystallised but still gave a yellow colour when ammonia was added to the diazotisation mixture. Further, the addition of ferric chloride to destroy the adrenaline present (as directed by Friend) did not alter the colour ratios obtained. This test is also described by Sansoni [1930] who states that it is the most sensitive and the easiest to employ, and although we were at a loss to account for its failure further attempts to utilise it were abandoned.

The remaining tests which were satisfactory qualitatively and appeared worthy of further investigation were Folin's test, the ammonium molybdate

test, and three oxidation reactions using ferric chloride, potassium iodate and potassium persulphate respectively as oxidising agents. Two of the latter were subsequently eliminated and will be dealt with first.

The ferric chloride reaction.

This reaction is characterised by the formation in slightly acid or neutral solution of a transient grass-green coloration, which changes to red on standing or by careful addition of dilute alkali. It is said that the more acid the solution the more fugitive and less marked is the green colour produced.

It was found that with pure adrenaline the green colour took about 10 minutes to change to red but that the reaction took place rapidly on boiling. A trial test was made using 2 cc. of a 1:100,000 adrenaline solution and 1 cc. of a 0·05 % $FeCl_3$ solution. The green colour produced on mixing was hardly discernible, but on boiling the solution and cooling, a colour of 1·8 red, 3·3 yellow Lovibond tintometer units was observed in a 1 cm. cell. The measurement of this orange-red colour seemed promising at the time but it was afterwards found that dilute $FeCl_3$ solution alone gives a somewhat similar colour on boiling, making the quantitative application of the test impossible. Further, it was found that suprarenal cortex extracts which contained no adrenaline gave definite reactions with this test.

It may be noted that alcohol does not interfere with this test and that if the colour obtained by addition of alkali be examined under ultra-violet light, using a Wood's screen, a very marked yellow fluorescence is observable very similar to that subsequently to be described under the ammonium molybdate test. The colour produced by adrenaline-free suprarenal cortex extracts does not show this property. The test is a very sensitive and reliable qualitative one for adrenaline but does not lend itself to quantitative application.

The potassium iodate reaction.

This test was originally applied quantitatively by Hale and Seidell [1911] who state that the method gives results 30 % below the biological value. Scoville [1920] who investigated the test much more fully found that the orange-red colour produced in neutral solution becomes reddish-violet in the presence of acid (acetic or hydrochloric) and prefers to do the test in acid solution (0·015 % hydrochloric acid) using an optimum temperature of 38° for 15 minutes. The method compares satisfactorily with the biological method provided the acidity of the original solution is known and reducing substances such as bisulphites (sometimes added as preservatives) are absent. By this method it is possible to estimate concentrations of adrenaline greater than 1 in 100,000 and the test is of about the same order of sensitivity as the persulphate test. Moreover, suprarenal cortex extracts free from adrenaline give no colour and the reaction should prove capable of measuring adrenaline in such extracts with a high degree of accuracy.

As, however, the reaction is essentially very similar to the persulphate reaction which is described later and as the latter is easier to use, the test was not further investigated.

The ammonium molybdate reaction.

This test, described by Rae [1930], depends on the presence of a catechol grouping in the adrenaline molecule. On mixing 1 cc. of adrenaline solution with 2 cc. of 10 % ammonium molybdate solution a yellow colour instantly develops and is permanent for several hours.

It was found that this test, while not so sensitive as the persulphate reaction, could be used in pure solutions for the quantitative estimation of adrenaline down to a dilution of about 1 in 20,000. Unfortunately when applied to concentrated gland extracts it gives very misleading results even when allowance is made for the yellow tint due to the extract alone.

The colour-concentration curve obtained with pure adrenaline is a straight line.

Other points noticed were that alcohol does not affect the reaction, except that too strong a concentration will precipitate the ammonium molybdate and also that the test cannot be applied to urine owing to the presence of other phenolic substances.

Paget [1930] uses this test to differentiate adrenaline and adrenalone, since, although both give an orange colour, the addition of sodium hydroxide discharges this colour to a greenish fluorescence with adrenaline but not with adrenalone. When observed under ultra-violet light adrenaline was found to give a very strong greenish-yellow fluorescence with Paget's test and this constitutes a very sensitive qualitative test for adrenaline, less than 1 in 2,000,000 being detectable. The colours given by adrenaline-free suprarenal extracts show no such fluorescence but the test could not be applied quantitatively without special apparatus.

Folin's test.

Folin's reagent [Folin *et al.*, 1912; 1913] was originally devised for the colorimetric determination of uric acid and many modifications of the reagent and technique employed have been suggested for the determination of uric acid in blood. In this connection Folin and Trimble [1924] attribute the inconsistencies met with by various workers to the presence of varying traces of molybdenum in the sodium tungstate used and describe a method for preparing the uric acid reagent free from such impurity. The sodium tungstate used by the present workers gave no reactions for molybdenum when tested by the xanthate test, and the reagent was therefore made without further treatment as directed by Folin and Trimble.

Although not specific for adrenaline this test is extremely sensitive, being about 10 times more sensitive than the persulphate reaction. The colour produced is a deep blue very similar to that given by uric acid, but it is said to be 3 times as sensitive for adrenaline as for uric acid.

The test has been applied to the estimation of adrenaline by Maiweg [1922] who is also of our opinion that the method gives too high results when applied to suprarenal extracts. Baker and Marrian [1927], in a fuller study of the test, estimated adrenaline in protein-free trichloroacetic acid extracts of suprarenal glands and calculated the experimental error as about 7 %.

Our first attempts to utilise this reaction were carried out as follows. 1 cc. of 1 in 100,000 adrenaline solution was added to 0·5 cc. Folin's reagent and 0·5 cc. saturated Na_2CO_3. The temperature was kept constant at 21°, but even with this precaution results could not be repeated. Elevation of the temperature to 37° resulted in a quicker development of the colour but the same inconsistencies were noticed.

These anomalies were found to be due at least partly to the metal cell used for matching the colours, since, as with the persulphate test, traces of copper dissolved from the metal markedly altered the reaction velocity. This, however, seems to be a peculiarity of the adrenaline test since the colour given by uric acid does not behave in the same way. The matter was not investigated further but the use of an all-glass cell was strictly observed.

Another difficulty encountered was the formation of crystalline precipitates after the mixture had been standing 10 to 15 minutes, the trouble being even worse at 37·5°. Some of the precipitate was removed and analysis showed it to be lithium phosphate which presumably only appears slowly owing to the slow breakdown of the phosphotungstic acid complex. It was found, however, that the use of only half the original quantity of Folin's reagent largely overcame this difficulty, as 45 minutes then elapsed before any precipitate was observed. In spite of these alterations no satisfactory results could be obtained with the test until the importance of the degree of alkalinity of the reaction mixture was discovered. It was found that, owing to the combined buffering action of the carbon dioxide evolved and the phosphates already present, the amount of sodium carbonate added brought the mixture to about p_H 8 only, and it was found necessary to make the reaction at least alkaline to thymolphthalein in order to obtain satisfactory results.

The use of sodium hydroxide as the alkali was therefore adopted, and while this causes the colour to fade rapidly if used in too great excess very consistent results are obtained if the following procedure is adopted. The amount of sodium hydroxide necessary is, of course, partly dependent on the Folin's reagent used and it is suggested that each sample should be titrated to thymolphthalein. In calculating the strength of the alkali to be employed an extra 2 % sodium hydroxide should be added to ensure the required alkalinity of the reaction mixture, *i.e.* if titration shows 0·25 cc. Folin's reagent equivalent to 0·75 cc. of 3 % NaOH, use 0·75 cc. of 5 %. This small excess of alkali is quite safe and does not cause any fading.

We have found that the use of sodium hydroxide as the only alkali constitutes a great advantage both in the determination of adrenaline and of uric acid. Not only does it enable remarkably constant figures to be obtained,

but the colour develops very much more rapidly, the maximum being reached within 3 minutes. The colour is just as stable as when sodium carbonate is used and no troubles are caused by the precipitation of phosphates or by the evolution of carbon dioxide. In view of the fact that many workers find the addition of sodium cyanide advantageous we attribute many of the discrepancies obtained with this test to the question of the p_H, not only in the estimation of adrenaline but also in the estimation of uric acid.

The final modification of this test adopted was as follows. Mix 1 cc. of an adrenaline solution of a concentration between 1 in 100,000 and 1 in 1,000,000 with 0·25 cc. Folin's reagent followed by 0·75 cc. of 5 % NaOH. The colour develops in a few minutes at room temperature and the maximum value is observed in a 1 cm. all-glass cell. The authors found that 0·01 mg. of adrenaline under these conditions gave a colour of 10·6 blue, 1·4 yellow Lovibond tintometer units. With pure solutions of adrenaline a straight line is obtained for the colour-concentration curve.

The potassium persulphate reaction.

Ewins [1910] described this test as the most sensitive reaction for adrenaline (giving a lower limit of 1 in 5,000,000). He also examined the effect of persulphate on various bases allied to adrenaline and found that the oxidation products such as aminoacetocatechol and its derivatives did not give the reaction. When applied to suprarenal extracts which were free from adrenaline we found that this test gave no colour and it was decided to investigate it more thoroughly in order to establish it on a quantitative basis.

Preliminary trials soon showed that Ewins had rather overestimated the sensitivity and that for quantitative measurement the adrenaline content should be not less than 1:100,000. The test was carried out by mixing 1 cc. of the solution under test with 1 cc. of 0·2 % $K_2S_2O_8$. The concentration of the adrenaline solution was kept constant at 1:10,000 and was made by diluting Liq. Adrenalin. Hydrochlor. B.P. 1914.

Effect of temperature.

The effect of variation of temperature was first studied. The colour was measured in a 1 cm. cell in the Lovibond tintometer at intervals, and the intensity plotted against the time (see Figs. 1 and 2), at temperatures of 18° and 37·5° respectively. It will be noticed that the maximum colour developed is slightly greater at 37·5°, being about 7·6 red, 3·4 yellow units and that the time required to reach this is much less at this temperature, actually about 25 minutes instead of the 55 minutes required at 18°. Further, the colour is stable over a longer period at 37·5°. The red tint is about double the yellow, but both tints are produced at about the same rate although the yellow starts to fade slightly before the red. When the red does fade, however, it does so much more quickly, especially at 37·5°.

Further experiments showed that at 0° the colour hardly develops at all, whilst at higher temperatures, 60° and 100°, although the colour appears

rapidly, it also fades very rapidly. Attempts to prevent this rapid fading by sudden cooling in a freezing mixture were not very successful and in no case was the colour obtained as high as that at 37·5°.

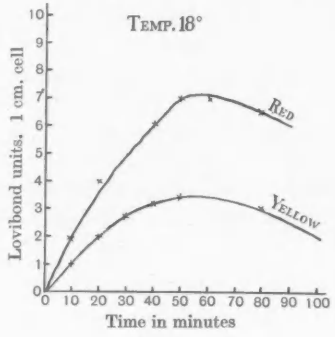


Fig. 1.

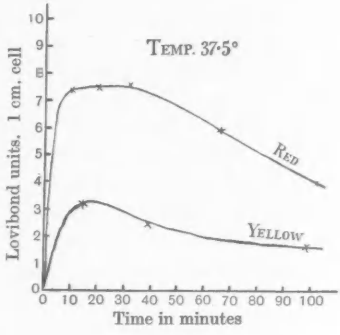


Fig. 2.

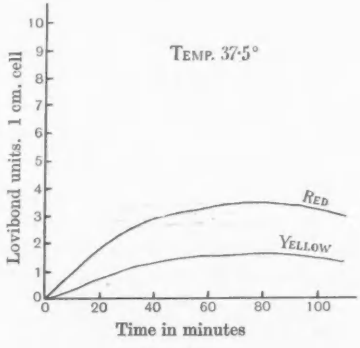


Fig. 3.

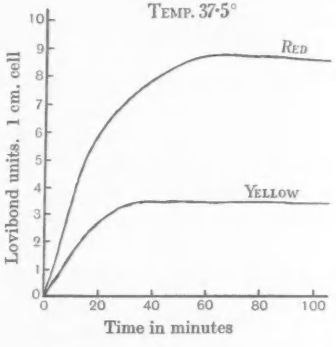


Fig. 4.

Fig. 1. 1 cc. 1 : 10,000 adrenaline (HCl). 1 cc. 0·2 % potassium persulphate.

Fig. 2. 1 cc. 1 : 10,000 adrenaline (HCl). 1 cc. 0·2 % potassium persulphate.

Fig. 3. 1 cc. 1 : 10,000 adrenaline (H_2SO_4) solution. 1 cc. 0·2 % potassium persulphate.

Fig. 4. 1 cc. 1 : 10,000 adrenaline (H_2SO_4) solution. 1 cc. 0·2 % potassium persulphate, 1·0 % sodium chloride.

Effect of chlorides.

When an attempt was made to confirm the above results, using a freshly made solution of adrenaline sulphate, very much lower figures were recorded. Thus, at 18° the colour development was almost nil, although it was higher at 37·5° (see Fig. 3). The original figures were confirmed, however, by diluting some freshly made Liq. Adrenalin. Hydrochlor. B.P. 1914 and it was obvious therefore that either the chloroform or the sodium chloride present in the B.P. liquor, or perhaps both, was responsible for the increased colour.

Experiment showed that the presence of chloride was the important factor and the effect of the addition of 1·0 % of sodium chloride to the reagent, using a 1 in 10,000 adrenaline sulphate solution, is shown in Fig. 4. This curve showed an improvement on all the previous work since not only was the maximum colour higher but it was established in a shorter time. Further, the colour was stable over a very much longer period. This stabilising effect of chlorides has been utilised in all the subsequent work, 1·0 % of sodium chloride being added to the reagent.

Effect of calcium chloride.

It has been noted by various workers that very dilute solutions of adrenaline in tap water turn pink much more rapidly than similar solutions in distilled water.

Two explanations suggested were:

(*a*) the chemical effect of dissolved substances in tap water;

(*b*) the buffering effect of tap water.

At first the conclusion was reached that the calcium present in the tap water was responsible for this phenomenon since the addition of 0·2 % $CaCl_2$ to the persulphate reagent resulted at 18° in a quicker establishment of the maximum colour. The only other observable effects were that slightly less yellow was produced and that the stabilising effect of the sodium chloride largely disappeared at 37·5° although it was retained at room temperature.

A modification of the test using a reagent containing 0·2 % $CaCl_2$, 1·0 % NaCl, 0·2 % $K_2S_2O_8$ was tried, but it was discovered that the reduction of the calcium chloride from 0·2 % to 5 parts per million produced no different results. We had to assume, therefore, that the effect of calcium chloride was either catalytic in nature or that it was entirely fortuitous and our attention was turned to a consideration of the p_H values during the reaction.

Effect of p_H value.

It is well known that the change of colour of adrenaline solutions brought about by oxidising agents is less rapid in acid solution than in neutral solution and more rapid in alkaline solution but we have not been able to find any record of an investigation of the effect of p_H upon the maximum colour produced by any given quantity of adrenaline or upon the time necessary for the production of that maximum coloration. This is a factor of great importance if the test is to be employed from the quantitative point of view, for variation of the p_H value of the reaction mixture was found to produce widely differing results.

Normally the p_H of the mixture was about 4·6, and the maximum colour was obtained in 30 minutes, but after the addition of acid to p_H 2 the maximum colour was not reached even after 7 hours. Attempts to determine the optimum p_H value for the reaction were at first unsuccessful, but this was traced to an increase in the p_H of the mixture as the reaction proceeded. It was therefore

necessary to incorporate a buffer in the reagent and several were tried, but those containing organic acids, especially citric acid, were excluded. The ordinary phosphate buffers were adopted, and the p_H values of the reaction mixtures were determined electrometrically using the quinhydrone electrode. Caution was necessary since the presence of an oxidising agent resulted in the cell becoming more acid on standing, and readings had to be taken immediately after adding the quinhydrone in order to obtain consistent results.

The time required to establish the maximum colour at 22° was plotted against the p_H value, keeping the adrenaline constant (1 in 10,000) and this curve is shown in Fig. 5. It clearly shows the optimum p_H value to be at p_H 5·5 and that if the mixture is either more acid or alkaline than this a very much longer time is required to establish the maximum colour.

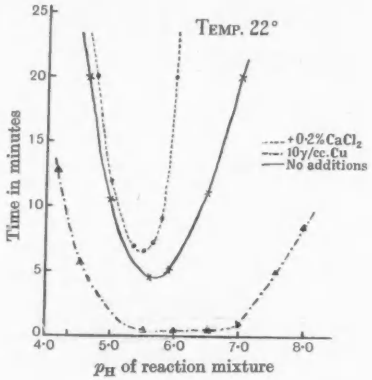


Fig. 5.

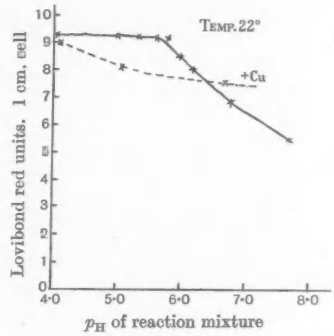


Fig. 6.

Fig. 5. Relation between time required to establish maximum colour and p_H of mixture (adrenaline 1 : 10,000). All in phosphate buffer.

Fig. 6. Effect of p_H on maximum colour. 1 cc. 1 : 10,000 adrenaline (HCl) solution. 1 cc. 0·2 % $K_2S_2O_8$, 1·0 % NaCl in phosphate buffer.

In Fig. 6 the maximum colour is plotted against the p_H value. It should be noted that only in solutions more alkaline than p_H 5·8 is there any reduction of the maximum colour so that fortunately in all our previous work, where the p_H happened to fall about 4·6, only the time required and not the actual maximum colour was liable to variation.

Also in Fig. 5 is given a similar curve in which 0·2 % $CaCl_2$ was present. This curve, although almost the same as that obtained in the absence of calcium, rises very sharply at p_H 6·0, and 24 hours are required at p_H 6·1 for the development of the maximum colour. This point coincides with the appearance of a precipitate of calcium phosphate. Thus it is obvious that no catalytic effect is associated with the calcium ion and the use of calcium chloride in the reagent was discontinued.

Catalytic effect of copper.

A chance observation emphasised the fact that copper will catalyse reactions of this kind. It was found that the colour developed more quickly in a metal cell than in an all-glass cell and this was found to be due to the dissolution of minute amounts of copper from the imperfectly plated framework of the cell. The curves obtained in the presence of 10 parts per million of copper (as sulphate) are shown in Figs. 5 and 6, using as reagent 0·2 % $K_2S_2O_8$ and 1·0 % NaCl in phosphate buffer solution. The catalytic effect results in the maximum colour being developed in less than half a minute over a wider p_H range (5 to 6). Fading is, however, much more marked although it does not occur in solutions more acid than p_H 4. This catalytic effect is intimately connected with the stability of the persulphate reagent, which probably functions in a similar manner to hydrogen peroxide. Other heavy metals such as mercury, cobalt, nickel and manganese also show some catalytic effect but not to the same degree as copper.

Iron on the other hand shows a slight inhibitory effect.

Colour-concentration curve.

As a result of the above work the following reagent was adopted and should be made with A.R. materials: 0·2 % $K_2S_2O_8$, 1·0 % NaCl, 0·239 % Na_2HPO_4, $12H_2O$, 0·937 % NaH_2PO_4, $2H_2O$. This solution should have p_H 5·5, and is stable if kept away from the light and in a cool place, but caution must be observed since when kept for some weeks at room temperatures the reagent tends to become more acid in spite of the buffer present, presumably owing to the release of sulphuric acid by the decomposition of the persulphate.

The colour-concentration curve for pure adrenaline (using the above reagent) is given in Fig. 7. Both the red and yellow tints give straight lines and it should be observed that both curves meet the horizontal axis at a point

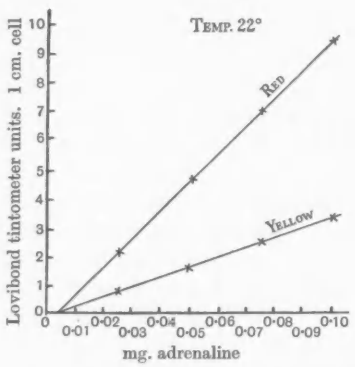


Fig. 7. Colour-concentration curve persulphate test. 1 cc. adrenaline solution (HCl). 1 cc. 0·2 % $K_2S_2O_8$, 1 % NaCl in phosphate buffer at p_H 5·5.

corresponding to 0·005 mg. of adrenaline. This point represents the limit of sensitivity of the test.

Application of the test to gland extracts.

Having thus determined the most favourable conditions for carrying out the test in pure solutions the method was applied to gland extracts. It was found that the colour did not develop so rapidly as in pure solutions, requiring about 30 minutes at 22°, probably because of the greater amount of easily oxidisable organic material in such an extract. The addition of a trace of copper sulphate enables the test to be carried out in less than 5 minutes but the results are less accurate and should not be used except as a preliminary indication.

The method recommended for gland extracts is as follows. Adjust the p_H of the extract to 5·4 (methyl red, external indicator). Then to 1 cc. add 1 cc. of the potassium persulphate reagent and immediately measure the colour (red units) in a tintometer using a 1 cm. all-glass cell. Then place the cell in the thermostat at 22° and read the colour again after 30 minutes. The increase of the red units is then compared with that given by a standard solution of adrenaline treated in a similar manner. The method can obviously be modified for comparison in a colorimeter.

The test is seriously interfered with by alcohol of which even 1·0 % affects the time required to produce the maximum colour. It is recommended that alcoholic solutions should be evaporated *in vacuo* making sure that the solution is more acid than p_H 5 and not allowing the temperature to rise above 30°. When free from alcohol, the residue is made up to the original volume with distilled water, and then tested as usual. No loss of adrenaline occurs under these conditions.

Comparison of tests on other substances.

The three tests found suitable for estimating adrenaline in pure solutions, *viz.* molybdate, persulphate and Folin's tests, were applied to a variety of organic substances which might cause interference and the results are given in Table I.

It will be noticed that Folin's test is not specific for adrenaline and, besides uric acid, most easily oxidisable substances give some blue colour, especially the aromatic polyhydroxy-compounds.

The molybdate test is, however, much better, tannic acid, and pyrogallol being the only other substances tried which gave the reaction. This, of course, is to be expected as they contain the catechol grouping. The fact that this test indicates the catechol group also means that substances closely allied to adrenaline, but without its pressor activity, may be the source of error encountered on applying this test to suprarenal extracts.

The persulphate test is the most specific test for adrenaline and (of the substances examined) gives a positive test only with metol and *p*-anisidine

Table I.

Test substance	Ammonium molybdate	Potassium persulphate	Folin's
Adrenaline	Orange-yellow	Red	Deep blue
Histamine	Nil	Nil	Nil
Liquid extract of ergot	Very slight pink	Nil	Prussian blue
Tyramine	Nil	Nil	Nil
Metol. OH⟨⟩$NHCH_3$	Nil	Red (on boiling only)	Intense blue
Salicylic acid	Yellow on warming; discharged by NaOH	Slight yellow	Nil
Tannic acid	Reddish orange; no u.v. fluorescence	Slight yellow	Intense blue
Tyrosine	Nil	Nil	Very slight blue
Trypsin	Very slight yellow	Nil	Blue
Vanillin	Nil	Nil	Very slight blue
Pyrogallol	Orange (as tannic acid)	Orange-brown on heating	Intense blue
o-Cresol	Very slight yellow on heating	Yellow on heating	Very slight blue
Salicylic aldehyde	Slight yellow on heating	Nil	Green-yellow precipitate on heating
Uric acid	Nil	Nil	Intense blue
Meat extract	Nil	Nil	Blue
Urine	Nil	Nil	Intense blue
p-Anisidine	Nil	Red on boiling	Nil
Phenacetin	Nil	Nil	Nil
Aniline	Nil	Slight purple	Nil

and then only on boiling. This test seems to depend on the presence of a primary or secondary amino-group in the side-chain and an —OH group in the *para*-position in the benzene ring. If, however, in addition to these groups a ketone group is present in the side-chain no reaction is obtained.

Ewins [1910] found that the following bases allied to adrenaline will give a positive persulphate reaction: arterenol (dihydroxyphenylethanolamine), dihydroxyphenylethylamine and epinine (dihydroxyphenylethylmethylamine), whereas the following will give no colour: homorenon (ethylaminoacetocatechol), methylaminoacetocatechol, ephedrine.

It should be noted that although some of these bases give the persulphate reaction, those which do so possess marked physiological action very similar to, but weaker than that produced by adrenaline. The corresponding ketonic oxidation products on the other hand do not give the colour test and do not generally possess physiological action much greater than that of catechol and its simple derivatives.

Comparison of colour tests with the biological method.

A comparison of the colour tests with biological results was made in order to prove definitely the impressions gained from previous work, *viz.* that Folin's and the molybdate tests were unreliable for the estimation of adrenaline in concentrated suprarenal extracts.

A sample of suprarenal cortex extract was taken and known quantities of standard adrenaline solution were added giving four extracts with upper and

lower limits of adrenaline at about 1 in 5000 and 1 in 100,000 respectively. The adrenaline was then determined colorimetrically in each extract by the ammonium molybdate, potassium persulphate and Folin's tests. Samples were assayed biologically at the same time.

Table II summarises the results obtained, the corresponding curves being shown in Fig. 8.

Table II. *Adrenaline in suprarenal extracts.*

Results as parts per 10,000.

	Extract "A"	"B"	"C"	"D"
Adrenaline actually present assuming "A" to contain 0·1 parts per 10,000	—	0·278	1·01	1·91
Persulphate test	<0·1	0·29	1·01	1·98
Folin's test	0·61	—	2·02	3·04
Molybdate test	1·5	3·9	5·4	6·7
Biological test	0·11	0·23	0·78	1·7

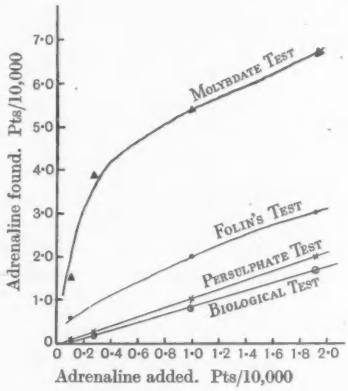


Fig. 8. Comparison of tests on gland extracts.

It will be seen that neither Folin's test nor the molybdate test gives reasonably correct results with gland extracts. The error of Folin's test is less than that of the molybdate test and in some cases might be very much less than that shown in Table II, since some samples of suprarenal extract gave much less colour with Folin's reagent, although of the same adrenaline content. The increase from sample "C" to "D" shown by Folin's test is in good agreement with that expected. Since such abnormal samples are always liable to occur, the use of Folin's test for quantitative work must be abandoned in spite of its great sensitivity, but negative results can be safely interpreted as indicating absence of adrenaline.

The results given by the persulphate test are as close as can be expected to the biological figures and are extremely close to the values anticipated, the

increases from samples "B" to "C" and from "C" to "D" being exactly the amounts actually added.

Attempts were made to use the persulphate test at dilutions below 1 in 100,000, and it was found possible by utilising the Klett colorimeter to carry out determinations with a degree of accuracy comparable with that of the biological method.

The estimation of adrenaline in glands.

It must be remembered that the suprarenal gland extracts described in this paper are of high concentration and naturally give the extremes of divergence of the various tests for adrenaline. In the quantitative determination of adrenaline in the suprarenal glands themselves, we obtained satisfactory results by using the protein-free trichloroacetic acid extracts described by Baker and Marrian [1927], who make use of Folin's reagent.

It is important, however, not to neutralise the filtrate before adding the reagents (Folin's or persulphate). Adrenaline is quite stable in trichloroacetic acid but if the solution is neutralised with sodium hydroxide, as the p_H approaches a value of about 3, a colour reaction takes place, a red colour forming which quickly fades to a fluorescent yellow. This results in the destruction of a large part of the adrenaline, but can be avoided by adding first the reagent and then afterwards the requisite quantity of alkali, allowing of course for the effect of the volume added on the dilution of the colour. Ox suprarenal gland by the persulphate test applied in this way showed an average adrenaline content of 3·3 mg./g. fresh gland, with 2 mg./g. in the cortex and 9 mg./g. in the medulla. The filtrates obtained from the trichloroacetic acid precipitation of defibrinated blood showed no adrenaline by the persulphate test. Addition of known amounts of adrenaline to the blood and subsequent precipitation showed that the persulphate test gave correct results (2 % loss) but the Folin's test gave results which were 25 % too high.

Summary.

1. The need for a rapid and accurate quantitative test for adrenaline in highly concentrated suprarenal gland extracts led to the investigation of many of the colour tests described in the literature. Some of these are unsatisfactory even from a qualitative point of view; others are non-specific. The latter criticism applies to the ferric chloride, ammonium molybdate and phosphotungstic acid (Folin's) tests, although the latter is extremely sensitive, and gives good results with comparatively pure adrenaline solutions if applied in accordance with the slightly modified technique described in this communication.

2. The potassium persulphate test has been investigated more thoroughly and although it is not as sensitive as Folin's test it gives results comparable with those obtained by the biological test when the necessary conditions, including control of temperature and p_H, are observed.

3. Preliminary work upon the application of the persulphate test to the determination of adrenaline in the suprarenal glands themselves indicates that it is of sufficient accuracy for this and similar purposes.

We are indebted to Mr F. Wokes for carrying out the biological tests.

REFERENCES.

Azzolini (1931). *Boll. Chim. Farm.* **70**, 665.
Bailly (1924). *J. Pharm. Chim.* (VII) **30**, 404.
Baker and Marrian (1927). *Biochem. J.* **21**, 1005.
Comessatti (1909). *Berl. klin. Woch.* **48**, 356.
Ewins (1910). *J. Physiol.* **40**, 317.
Folin and Denis (1912). *J. Biol. Chem.* **12**, 239.
—— Cannon and Denis (1913). *J. Biol. Chem.* **13**, 477.
—— and Trimble (1924). *J. Biol. Chem.* **60**, 473.
Friend (1923). *J. Biol. Chem.* **57**, 497.
Hale and Seidell (1911). *Amer. J. Pharm.* **83**, 551.
Maiweg (1922). *Biochem. Z.* **134**, 292.
Orru (1929). *Ann. Chim. App.* **19**, 239.
Paget (1930). *Bull. Sci. Pharm.* **37**, 537.
—— (1931). *Bull. Sci. Pharm.* **38**, 77.
—— and Lohéac (1928). *J. Pharm. Chim.* **8**, 159.
Rae (1930). *Pharm. J.* **125**, 451.
Sansoni (1930). *Officina*, **3**, 323.
Scoville (1920). *J. Ind. Eng. Chem.* **12**, 769.
Stuber, Russmann and Proebsting (1923). *J. Soc. Chem. Ind.* **42**, 739 A.
Viale (1930). *Bull. Soc. Ital. Biol. Sper.* **5**, 1163.

CCLIII. THE APPLICATION OF PHOSPHO-18-TUNGSTIC ACID (FOLIN'S REAGENT) TO THE COLORIMETRIC DETERMINATION OF CYSTEINE, CYSTINE AND RELATED SUBSTANCES.

I. THE REDUCTION OF PHOSPHO-18-TUNGSTIC ACID BY VARIOUS SUBSTANCES.

By JOSEPH W. H. LUGG.

From the Division of Animal Nutrition of the Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia.

(*Received September 12th, 1932.*)

Of the phosphomolybdates and phosphotungstates those containing eighteen atoms of the heavy metal to every P_2O_5, members of the 1–18 series in our present nomenclature, have been exploited most in colorimetric estimations on account of the ease with which their pale-coloured solutions may be reduced by certain substances to give intense blue, greenish, or reddish tints. Some use has lately been made of the corresponding arsenic compounds.

Wu [1920] arranges the 1–18 and 1–24 phospho-molybdates and -tungstates in order of increasing sensitiveness to reduction, as follows: phospho-24-tungstic acid, B-phospho-18-tungstic acid, A-phospho-18-tungstic acid, phospho-24-molybdic acid, and phospho-18-molybdic acid. He further states that acids of the 1–17 and 1–22 series are quite inert. Salts of the 1–17 and 1–22 series are formed when those of the 1–18 and 1–24 series respectively are treated with excess of alkali hydroxide or carbonate. Salts of phospho-17-tungstic acid are thus precipitated as small white crystals difficultly soluble in large excess of alkali. These salts, together with others containing much more phosphorus which are supposed to exist, are said to regenerate 1–18 and 1–24 acids exclusively on treatment with mineral acids.

Wu concluded that two forms, A and B, of phospho-18-tungstic acid exist, and that his B form may be identified with the 1–18 acid of Kehrmann [1887], who was the first to prepare such a compound. He notes however that Kehrmann probably prepared both forms. The A and B forms are said to yield the same 1–17 acid on treatment with alkali, and the 1–17 acid regenerates exclusively the B 1–18 acid on treatment with mineral acids.

Some doubt has been thrown upon the existence of the A form by Folin and Marenzi [1929], who suggest the presence of molybdenum impurity in Wu's sodium tungstate and that the alleged A acid may have been a molybdo-tungsten complex.

Folin and Looney [1922] made use of the Folin and Denis [1912] uric acid reagent to determine the cystine content of protein hydrolysates. The uric acid reagent is essentially phospho-18-tungstic acid prepared by treating sodium tungstate with phosphoric acid, and if Wu's findings are correct one would expect it to contain both the A and B forms. The method is based upon the observation (apparently unpublished) of T. Zucker, that cystine *plus* sodium sulphite gives colours with the uric acid and phenol reagents, whereas cystine alone does not, and sulphite alone develops a comparatively feeble colour. Heffter [1907] had discovered that cystine may be reduced by sulphite to give cysteine.

Since 1922 many modifications of the Folin-Looney method have appeared. Hunter and Eagles [1927] warned against having standard and unknown at even slightly different degrees of alkalinity. Folin and Marenzi [1929], following the lead of Folin and Trimble [1924], carefully removed molybdenum from the reagent to avoid reactions with tyrosine, tryptophan and phenolic bodies generally. They also improved the technique in some directions. Rimington [1930], working upon an observation made by Folin, added urea to prevent precipitation of the 1–17 tungstate; an event that frequently ruins estimations under the Folin-Looney and Folin-Marenzi procedures. Folin and Marenzi themselves had tried to overcome this difficulty by adding large amounts of lithium salt to the reacting mixture. In their mixtures the solubility of lithium carbonate is so grossly exceeded that it is surprising its precipitation does not occur more frequently than it actually does. Tompsett [1931] reduced the alkalinity of the reacting mixture and thereby avoided precipitation of the 1–17 tungstate, reduced the effect of slight changes in alkalinity upon the colour developed, and greatly reduced interference by uric acid. Most of the solutions (*e.g.* hydrolysates of tissues) which have to be analysed for cystine contain other substances which contribute to the coloration developed. Uric acid gives much colour in both the Folin-Looney and Folin-Marenzi procedures in absence of sulphite, and more in presence of it. Those soluble products arising from the digestion of carbohydrate with mineral acid at the boiling point also reduce the reagent. It has become customary to subtract the blank obtained in absence of sulphite from the colour obtained in presence of sulphite, and to call the nett result "cystine." Inasmuch as the coloration developed by extraneous substances may be increased when sulphite is present, and perhaps reduced by the very presence of cystine, this procedure is unjustified. Furthermore, cysteine reduces the reagent in absence of sulphite, and this seems to have been neglected in nearly all interpretations of the results, the blank obtained in absence of sulphite being calculated nearly always as "uric acid."

In his study of the alkaline decomposition of cystine, Andrews [1928] used the Folin-Looney procedure for the estimation of both cysteine and cystine. With some concern he noted that the colour developed in presence of sulphite sometimes exceeded that which would have been obtained if none of the cystine had been destroyed, and ascribed this remarkable result to the presence of

some unknown strongly reducing substance produced in the treatment of cystine with alkali. His expectations were really based upon the accepted belief that sulphite reduces cystine to cysteine under the conditions of the cystine estimation. The fate of the cysteine during its oxidation by the reagent seems not to have interested anybody.

There is reason to believe that any sufficiently soluble sulphhydryl compound will reduce the reagent, and the same may be said of any disulphide which will react with sulphite to form a sulphhydryl compound. It is clear then that the reaction with phospho-18-tungstic acid is not only unspecific for cysteine among the sulphhydryl compounds, but is further unspecific for sulphhydryl compounds themselves. This lack of specificity has led many to abandon phosphotungstic acid in favour of a far more specific colour reaction discovered by Sullivan [1926], and, in turn, the enormous difficulties attending the application of Sullivan's reaction to quantitative analyses have thrown the list of estimations in the literature into a rather chaotic state.

The present paper constitutes an analysis of the phospho-18-tungstate colour reactions, and includes among other things, investigations of the nature of the reaction between cysteine and the reagent, the reactions between cystine and sulphite, and the degree to which the reagent is reduced by substances other than cysteine but likely to occur in a mixture to be analysed for cysteine or cystine.

Experimental.

The phospho-18-tungstic acid was prepared free from molybdenum according to the method of Folin and Marenzi [1929]. Some of the reducing substances examined were carefully prepared in this laboratory. I am indebted to Mr H. R. Marston of this Division for the supply of cystine.

The coloration developed by a reducer may vary with the time allowed for the reaction, the concentration and amounts of the reacting materials, the p_H of the reacting mixture, salt concentration and type, the order in which the materials are added, and sometimes with small amounts of substances which may react with a particular reducer under examination. With the more powerful reducers the effect of temperature seems to be almost nil.

Except in alkaline solutions, the order of adding the different materials has but little effect upon the colour developed in 5 minutes. In alkaline regions slightly greater colorations result if the reagent is first made alkaline and the reducers are then added, than if the reagent and reducer are first mixed and then made alkaline. This effect increases slightly with the time for which the alkaline reagent is allowed to stand, provided this is limited to a few minutes. Solutions containing cystine *plus* sulphite, cysteine, or ferrous salt must never be made alkaline before the reagent is added, or there will be a decided loss in coloration developed (*cf.* the Folin-Looney and Folin-Marenzi procedures for the estimation of cystine). Na_2S as reducer cannot be added to an acid solution and made alkaline afterwards. Such procedure would result in a loss of H_2S.

Fig. 1 expresses graphically the variation with p_H of the blue coloration developed by some of the reducers and mixtures, under conditions to be described. Different amounts of reagent were used in obtaining different curves, but for each curve the only important independent variable was the p_H. The free phosphate in the reagent acted as a buffer in certain regions but most of the buffering was done by acetate, citrate, and borate mixtures added for that purpose. In the more alkaline regions sodium carbonate was used to "neutralise" the reagent after the reducer had been added, and in the more acid ones sodium acetate was used. Temperature varied very little from 25°.

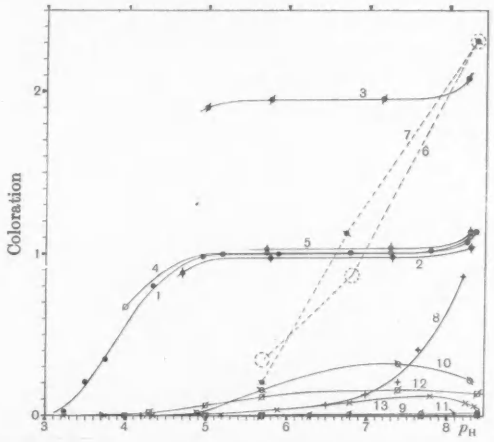


Fig. 1. 1, cystine + sulphite; 2, cysteine; 3, cysteine + sulphite; 4, Fe++; 5, Fe++ + sulphite; 6, Na_2S; 7, Na_2S + sulphite; 8, uric acid; 9, furfuraldehyde; 10, furfuraldehyde + sulphite; 11, laevulinic acid; 12, laevulic acid + sulphite; 13, sulphite.

Short of recording in a lengthy table the actual amounts of carbonate, citrate, acetate, *etc.*, used it would be impossible to state the actual salt concentrations, but it is believed that the reduction in the activity of the water in the final diluted solutions would correspond approximately with that observed in a 0·4 *M* NaCl solution. In so far as salt type and concentration may greatly influence the activities of the species taking part in the colour reactions, the point is however not without importance. Two sets of solutions were used in obtaining each curve. They differed only in that the members of the set used in estimating the coloration received reducing substance whilst the corresponding members of the other set, used in p_H determination, did not. p_H was determined colorimetrically with the aid of a wide range of indicators and recorded values may be in error by 0·1 unit or more in some instances.

The colour reactions were carried out in 100 cc. standard flasks. Colour development was allowed to proceed for 5–6 minutes in a volume not exceeding 30 cc. and not less than 15 cc., the volume being kept constant for any one

substance under investigation whatever the p_H or buffers employed. After this interval had elapsed the solutions were diluted to the mark with pure water and within 5 to 15 minutes were compared with some arbitrary and constant standard. It was found necessary to have some citrate present in alkaline solutions containing iron, tin, *etc.* to avoid formation of precipitates. Lithium sulphate was never added. The p_H beyond which one cannot proceed without precipitating the 1–17 tungstate is 8·5. The curves are for colorations obtained with:

1. 2 mg. (1/120 millimol.) of cystine *plus* 1 millimol. of Na_2SO_3 with 5 cc. of reagent.
2. 2·017 mg. (1/60 millimol.) of cysteine alone with 2 cc. of reagent.
3. 2·017 mg. of cysteine *plus* 1 millimol. of sulphite with 2 cc. of reagent.
4. 0·93 mg. (1/60 millimol.) of Fe^{++} alone with 2 cc. of reagent.
5. 0·93 mg. of Fe^{++} *plus* 1 millimol. of sulphite with 2 cc. of reagent.
6. 1·30 mg. (1/60 millimol.) of Na_2S alone with 10 cc. of reagent.
7. 1·30 mg. of Na_2S *plus* 1 millimol. of sulphite with 10 cc. of reagent.
8. 2 mg. (1/64 millimol.) of uric acid alone with 5 cc. of reagent.
9. 20 mg. (1/4·85 millimol.) of furfuraldehyde alone with 2 cc. of reagent.
10. 2 mg. of furfuraldehyde *plus* 1 millimol. of sulphite with 2 cc. of reagent.
11. 10 mg. (1/11·6 millimol.) of laevulic acid alone with 2 cc. of reagent.
12. 10 mg. of laevulic acid *plus* 1 millimol. of sulphite with 2 cc. of reagent.
13. 1 millimol. of Na_2SO_3 with 5 cc. of reagent.

In order that the figure might not be unduly complicated, many of the data have been left out of it, and some of these will be recorded below.

When carefully purified and fresh, pyruvic acid (10 mg.) does not reduce the reagent at any p_H. In acid regions the coloration developed in presence of sulphite is practically that of the sulphite itself, but in more alkaline regions the coloration is somewhat greater than that due to the sulphite alone. Pyruvic acid polymerises on standing and old, impure samples may reduce the reagent.

Creatinine only slightly reduces the reagent, the least reduction being observed in acid solutions. Quinol reduces the reagent strongly in alkaline regions and fairly well in acid solutions. Phenylhydrazine and "metol" are powerful reducers. Cuprous chloride reduces the reagent after the manner of ferrous salt. Ferric chloride *plus* sulphite gives a colour corresponding with that of the sulphite alone. Stannous chloride alone gives a slight colour in acid solution but a stronger one in alkaline. With sulphite the colour developed is much greater than mere addition would allow, and the effect is therefore reminiscent of that observed with furfuraldehyde or laevulic acid. Ethyl sulphide reduces only slightly in acid solution and rather more strongly in alkaline. It is very sparingly soluble. The addition of sulphite intensifies the colour somewhat. It is probable that the sample contained a trace of mercaptan or of a disulphide, or both. Oxalates, malates, and tartrates have no effect upon the reagent. Tetrathionates seem to be without effect.

Stability.

Colorations developed by cysteine, cystine *plus* sulphite, cysteine *plus* sulphite, ferrous salt, and ferrous salt *plus* sulphite are extremely stable in the region p_H 5·0–6·5. As a rule they increase by 1 or 2 % in the course of several hours and then begin to fall. Using 5 cc. of reagent at p_H 5·7, the coloration with 2 mg. of cystine *plus* 1 millimol. of Na_2SO_3 is reduced by only 5 % in 18 hours, whereas at p_H 8·35 the loss may be 30 %. After 48 hours the losses might be 20 and 50 % respectively. Below p_H 4·0 the development of colour by cystine *plus* sulphite is extremely slow and the depth of coloration increases considerably during several hours. The coloration developed by cysteine alone or ferrous salt, on the other hand, comes up rapidly in acid mixtures, but in the case of ferrous salt, with or without sulphite, the solutions fade during dilution and for some time thereafter. In acid solutions, colorations given by Na_2S increase considerably with time.

Colorations developed by sulphite alone, laevulic acid with and without sulphite and furfuraldehyde with and without sulphite increase relatively much more in the first few hours and fade much more slowly.

The coloration developed by quinol is curious in that it fades extremely rapidly. At p_H 6·5 for example, the loss may be 50 % within 30 minutes.

The coloration developed by uric acid alone progressively increases in acid solution during 18 hours and longer, whereas in alkaline solution it fades much more rapidly than in the case of cystine *plus* sulphite. Besides greatly increasing the depth of coloration with uric acid, sulphite improves the stability in alkaline solution.

The effects of varying the amounts of reagent, etc.

A series of cystine *plus* sulphite colorations developed by only 2 cc. of reagent, yielded a curve rather less than 2 % below the one in Fig. 1 over the range p_H 5·0–7·5. Below p_H 5 the curve approached the abscissa more steeply than the one in the figure and above p_H 7·5 the curve rose less steeply, thereby following more closely the curves for cysteine and ferrous salt (both also obtained with 2 cc. of reagent) in this region and down to p_H 5. The reverse variations from the curve in the figure were observed when 8 cc. of reagent were employed. The amount of sulphite could be doubled or halved without affecting the coloration developed with cystine by more than 2 %, provided the reactions were carried out above about p_H 5·2 and that sufficient time, the usual 5–6 minutes, was allowed for colour development. The rate of colour development however was greatly influenced by varying the quantity of sulphite, rate and quantity of sulphite decreasing together, and more so the lower the p_H. Below p_H 5 the curve became steeper as the quantity of sulphite employed was decreased. The shape of the curve below p_H 5 therefore depends largely upon the amounts of sulphite and reagent employed and the

time allowed for the reaction, as it tends to flatten out if the 5–6 minute interval is increased.

The colorations developed by cysteine and ferrous salt do not vary much with the amount of reagent, the effects at each end of the curves being less pronounced than they are with cystine *plus* sulphite.

On the other hand the coloration with sulphite alone increases with increasing amounts of reagent but not linearly. These remarks apply also to furfuraldehyde and laevulic acid, alone and with sulphite, but for uric acid alone the depth of coloration is nearly proportional to the amount of reagent over the entire p_H range.

The coloration developed by Na_2S in the 5–6 minute time interval increases with the amount of reagent when only a few cc. are used. With 5 or 10 cc. there is not much difference in the amount of colour developed at high p_H values.

The rate of colour development decreases as the volume of the reacting mixture is increased. With large volumes, full colour development may be attained with cysteine or ferrous salt in 5 or 6 minutes, but with cystine *plus* sulphite or with Na_2S this may not be so, particularly in acid regions.

Proportionality.

The depth of coloration developed by cysteine, cystine *plus* sulphite, ferrous salt, and Na_2S, the last subject to certain restrictions in the p_H range, is strictly proportional to the amount of reducer employed within the usual limits imposed by the nature of colorimetry, provided that the chosen p_H be maintained and that sufficient reagent be present. Thus in presence of 2 cc. of reagent, 4 mg. of cystine *plus* sulphite give approximately 3·95 times as great a coloration as is developed by 1 mg. of cystine *plus* sulphite at any fixed p_H in the range 5·0–8·4.

At any chosen p_H cysteine *plus* sulphite gives almost exactly (to within 1 %) twice the coloration developed by the cysteine alone. The curves showing this relationship may be seen in Fig. 1.

At any chosen p_H the depth of coloration developed by uric acid is far from proportional to the amount present, reagent concentration remaining the same. Thus with 2 cc. of reagent at p_H 8·4, 2 mg. of uric acid give a coloration only 20 % greater than does 1 mg. If sulphite is present and/or the amount of reagent be increased, a deeper coloration is developed and the poor proportionality is improved. Thus under the same conditions as before, but with 1 millimol. of sulphite present, 2 mg. of uric acid give a 40 % greater coloration than does 1 mg.

Other variables remaining the same, the depth of coloration developed by sulphite alone is approximately proportional to the amount of sulphite present.

Furfuraldehyde alone and laevulic acid alone, give colorations varying more or less directly with the amounts present when other variables are unaltered. The colorations developed in presence of a fixed amount of sulphite however are by no means proportional to the amounts present. There

appears to be no maximum with laevulic acid, but with 2 cc. of reagent at p_H 5·7 and in presence of 1 millimol. of sulphite, 3 mg. of furfuraldehyde give a greater coloration than any other amount. In presence of 1 millimol. of sulphite at p_H 5·7, 0·25 and 0·5 millimol. of $SnCl_2$ develop about the same degree of coloration.

Inhibition and enhancement of colour development.

Sufficient acetone, if added before reagent and sulphite, will completely inhibit the production of colour by cystine for some distance on either side of p_H 5·7. 2 mg. of cystine *plus* 1 millimol. of purified acetone-bisulphite compound will produce at p_H 5·7 about 80 % of the coloration obtained by using sulphite instead of acetone-bisulphite, and 1 millimol. of acetone-bisulphite will give only a fraction of the coloration developed by 1 millimol. of sulphite alone.

In presence of sufficient furfuraldehyde (*e.g.* 150 mg.) cystine *plus* sulphite fails to reduce the reagent appreciably.

When a fairly large amount (1 or 2 millimol.) of cadmium chloride is added to a solution containing cystine and followed by sulphite and reagent, the coloration develops extraordinarily slowly so that at the end of 5 minutes only 60 % of the full colour value may be attained at p_H 5·7. $CdCl_2$ also reduces the depth of coloration developed by laevulic acid *plus* sulphite and by furfuraldehyde *plus* sulphite.

Zinc chloride in quantity (4 millimol.) will reduce the coloration developed by cystine *plus* sulphite at p_H 5·7 by several %. 2 millimol. of $ZnCl_2$ do not seem to alter the coloration given by sulphite alone. The coloration developed by cysteine is very slightly decreased and that by ferrous salt not at all. 2 millimol. of $ZnCl_2$ will reduce the coloration given by 10 mg. of laevulic acid *plus* sulphite by as much as 35 %. The coloration given by furfuraldehyde *plus* sulphite is also greatly reduced.

Mercuric chloride does not alter the coloration given by sulphite alone, but in the presence of even such a small amount as 0·1 millimol. of $HgCl_2$, cysteine alone fails to develop a trace of colour with the reagent at p_H 5·7, and cystine *plus* sulphite yields only the colour which would be expected of the sulphite alone. The effect extends beyond p_H 5·7 in either direction. When solutions of cysteine hydrochloride and $HgCl_2$ are mixed, a white precipitate of the mercaptide forms immediately. It is readily soluble in citrate buffer at p_H 5·7 and in strong acids. $HgCl_2$ does not affect the coloration developed by ferrous salt. 0·2 millimol. of $HgCl_2$ will reduce the coloration given by laevulic acid *plus* sulphite by about 20 %. $HgCl_2$ gives a white precipitate with uric acid at p_H 5·7. The precipitate is not soluble in citrate buffer but is readily dissolved by sulphite, by thiosulphate, by $CdCl_2$, by $ZnCl_2$ and by large quantities of NH_4Cl.

The heavy metals do not appear to influence the colorations given by laevulic acid or furfuraldehyde alone to a marked extent.

The coloration developed by sulphite alone may be increased considerably in the more acid mixtures by addition of much salt. Thus at p_H 5·7, 50 millimol. of Na_2SO_4 may nearly treble the coloration developed by the sulphite.

Ammonium chloride in quantity (5 millimol.) at p_H 5·7 increases the rate at which the coloration given by cystine *plus* sulphite develops, whereas large amounts of citrate buffer rather diminish the rate. NH_4Cl does not influence the coloration developed by ferrous salt but it may increase that given by cysteine alone by about 1 %. 5 millimol. of NH_4Cl increase the coloration given by 10 mg. of laevulic acid *plus* sulphite by about 18 % at the same p_H. It also greatly increases the coloration given by furfuraldehyde *plus* sulphite.

NH_4Cl does not appear to influence the colorations developed by laevulic acid or furfuraldehyde alone to a marked extent.

Reduction of the reagent by mixtures of substances.

The type example is the reaction by which cystine may be estimated. Cystine alone does not reduce the reagent. Sulphite reduces but slightly. A mixture of the two reduces strongly and the final coloration is virtually proportional to the cystine present provided this is not infinitesimal and that sufficient reagent is employed. Assuming that the sulphite contributes its full individual share to the total coloration developed in presence of cystine, one may calculate the departure to be expected from linearity of depth of coloration with cystine, assuming that only a small amount of sulphite is involved in converting the cystine to the same degree each time into something that will reduce the reagent. The calculated departure from linearity is several times in excess of that actually found. In other words the sulphite appears not to contribute its own coloration completely.

The same phenomenon is more easily appreciated from the curves in Fig. 1, showing the colorations developed by ferrous salt in absence and presence of sulphite. Here it is obvious that superimposing a sulphite curve on one given by ferrous salt alone will not lead to a curve of the type given by ferrous salt *plus* sulphite. As the displacement between the curves for ferrous salt with and without sulphite is small, and about the same at all p_H values, it is believed to be due to the sulphite precluding aerial oxidation of the ferrous salt rather than anything else.

Under certain conditions of volume, *etc.* and in the presence of 2 mg. of cystine, 1 millimol. of sulphite, and 2 cc. of reagent at p_H 5·7, 10 mg. of pyruvic acid, 10 mg. of laevulic acid, and 2 mg. of furfuraldehyde increased the coloration developed, by 0, 1 and 1·5 % respectively; whereas if the colorations had been additive the increases would have been 1, 8 and 11 % respectively.

Similarly the products of acid digestion of carbohydrates have been found to contribute only a fraction of the coloration they develop with sulphite to the total coloration obtained when fair quantities of cystine, ferrous salt, or cysteine are present.

Analysis and discussion of the experimental data.

The effects noticed when NH_4Cl and the heavy metal chlorides are added to laevulic acid or furfuraldehyde in presence of sulphite are really enhancement and inhibition of the rate of colour development. If the colour reactions are allowed to proceed for 5 hours instead of 5 minutes, the effects mentioned are almost lost.

The same might be said of the effects these substances exert in the case of cystine and cysteine. The actions of $CdCl_2$ as an inhibitor and of NH_4Cl as an enhancer are certainly upon the rate of colour development rather than upon the final equilibrium that might be attained, though this also might be affected. It is not unlikely that the action of $HgCl_2$, apparently protection of the —SH group by mercaptide formation, is of the same type, the effect being so pronounced however that no noticeable colour is developed even after some hours. The effects of these three heavy metal chlorides thus increase with their tendency to behave as non-polar substances and this is the order of the appearance of the metals in the same sub-group of the periodic system.

It is commonly stated that bisulphite reacts with only the lower members of ketonic series to form bisulphite compounds. The increased colour development noticed when furfuraldehyde and laevulic acid reduce the reagent in presence of sulphite cannot be explained. It can hardly be due to bisulphite compound formation since, for example, if a large excess of furfuraldehyde be used, extremely little colour is developed. This inhibiting effect, however, like that of acetone, is believed to be due to bisulphite compound formation.

As Wu points out in his examination of the complex acids, the "reducibility" of these substances by most reducers is small. He concludes that very few of the heavy metal atoms in the whole bulky complex are reduced as a rule, and that phospho-18-tungstic acid loses one atom of oxygen when reduced by ferrous salt in alkaline solution.

The colour reaction may be written:

$$\text{reagent} + \text{reducer} \rightleftharpoons \text{reduced reagent} + \text{oxidised reducer} \quad \ldots\ldots(1),$$

but this form involves the assumption that a true equilibrium may be reached. Until some small amount of the reagent is reduced the oxidation potential of the reagent system is virtually infinite.

In referring to oxidation-reduction potentials of the reagent and reducer systems we really imply that the potential of the one system is linearly related to $ln \frac{[\text{reagent}]}{[\text{reduced reagent}]}$ and that of the other to $ln \frac{[\text{reducer}]}{[\text{oxidised reducer}]}$. These relationships might not apply, but assuming that they do it is not difficult to see why different reducers might give different amounts of colour and why weak reducers fail to develop their full colorations in presence of strong ones. When cysteine, ferrous salt, cystine *plus* sulphite or Na_2S (the last in alkaline solution) is the reducer, the balance represented by the arrows is well in favour of the right-hand side of the equation. With other substances the balance

seems to be in favour of the left-hand side. This is evidenced by the greater colour developed when more reagent is used, as in the case of uric acid; the lack of proportionality between colour and amount of reducer, the poor reduction effected by large amounts of potential reducer, *etc.* The actual magnitudes as well as the ratios of the forward and backward velocity constants must be important factors in some instances.

Cysteine and the other strong reducers certainly seem to achieve some sort of balance. At p_H 5·7 with 2 cc. of reagent, 1, 2 and 4 mg. of cystine *plus* sulphite or of cysteine alone, or 1 and 2 mg. of cysteine *plus* sulphite, give amounts of colour almost strictly proportional to the amounts of cystine or cysteine present. With 6 mg. of cystine *plus* sulphite or 6 mg. of cysteine or 3 mg. of cysteine *plus* sulphite, the departure from linearity is slight, but with say 10 mg. of cysteine or a corresponding amount of ferrous salt for example the departure is considerable (20 %). With 80 mg. of cysteine the departure is about 800 %. The relation between colour and amount of cysteine follows approximately the same trend at p_H 8·4, though the balance would appear to be rather more in favour of the right-hand side of the equation than it is at p_H 5·7.

The reagent is probably almost exhausted when such large amounts of cysteine are employed. Assuming that all the tungsten present in 2 cc. of reagent exists in the complex and that each molecule of the complex may lose only 1 atom of oxygen to cysteine or ferrous salt, then if 80 mg. of cysteine could reduce all the reagent the departure from linearity would be 670 % instead of 800 %. If Wu's findings are correct then at least 80 % of the tungsten present in the reagent employed here exists in the complex. But the balance cannot be so completely in favour of the right-hand side of the equation (*cf.* the departure with 10 mg. of cysteine) and hence it is possible that nearly 100 % of the tungsten exists in the complex.

It will be seen later that cysteine is oxidised to cystine by the reagent. Whatever the nature of the balance achieved by cysteine may be, it cannot at present be interpreted in known physico-chemical terms. The potential of a cysteine-cystine system at an inert electrode cannot be described by Peter's equation. In the equation that describes the potential,

$$E = E_0 - \frac{RT}{F} ln\,[\text{cysteine}] + \frac{RT}{F} ln\,[H^+],$$

the concentration of cystine finds no place at all, and attempts to explain it in terms of Wieland's theory of hydrogen acceptors have recently been discredited by Michaelis and Flexner [1928].

Those reducers which seem to establish an early balance with the reagent practically all give increasing colorations at p_H values increasing up to about 7·5. Above p_H 6·5 the predominating sulphite ion in a sulphite solution will be the $SO_3^=$ ion. With the exception of such substances as uric acid the colorations thereafter decline. This effect is offset by that observed with the stronger reducers as p_H increases above about 7·5. That the colorations with ferrous

salt and the other strong reducers should increase, strongly suggests that the reagent is subject to a different reduction from that occurring in the acid solutions or that the reagent is of different form in the two zones. The former possibility seems to be ruled out by the discussion on the extent to which 2 cc. of the reagent are reduced by 80 mg. of cysteine at p_H 5·7 and p_H 8·4. The latter possibility, if accepted, revives interest in Wu's claim that the 1–18-acid may exist in two forms A and B. The A form which is said to give nearly 30 % more colour than the B on total reduction is also said to be more easily reduced than the B form. With this in mind, the curves in Fig. 1 might suggest that the two forms are in equilibrium at any p_H, the B form predominating at low p_H and the A at high, though one would expect the weak reducers to give more instead of less colour at high p_H if this were so, and that a new equilibrium is only slowly attained when the active mass of one form is altered. This view is supported by the observations made on the effects of the order of adding the materials in alkaline solution.

The very rapid fading encountered with quinol as reducer cannot be explained. Excess bromine readily destroys the blue colour developed by the substances described in this paper, but H_2O_2 is usually almost without effect. Saturation with acetone sometimes causes complete bleaching in a few seconds.

The slow fading observed with cysteine, cystine *plus* sulphite, *etc.*, as reducers seems to be associated in some way with decomposition of the 1–18-acid. Its rate increases with the p_H and to some extent with salt concentration and type. Thus large amounts of $ZnCl_2$ or $CdCl_2$ very considerably increase the rate. The stock reagent used in all this work is buffered at about p_H 2·1 and remains stable for years. If some reagent is brought to p_H 8·4 and buffered it will be found to lose practically all its reducibility after some hours. Buffered at p_H 5·7 the reagent will "work" for a few days. Thereafter it loses its pale apple-green colour, which becomes pale sky-blue, and is not reduced by cysteine or cystine *plus* sulphite. Ferrous salt reduces it slightly giving a blackish-green tint. Nothing is precipitated from the reagent kept at p_H 5·7 even after standing for 5 months at room temperature. This form of decomposition of the reagent is not at present understood.

In reference to the colorations given by ferrous salt, cysteine and the other members of that group it has been concluded that the reducers are virtually fully oxidised to other substances by a relatively small excess of reagent, and from the colorations developed by ferrous salt during its oxidation to the ferric state one may associate a certain amount of colour with a certain amount of reduction.

In extreme alkaline regions and with plenty of reagent, the coloration given by Na_2S corresponds with the oxidation of both atoms of hydrogen in the H_2S molecule. Unless sulphite is present the solution is clouded by free sulphur and as the sulphite does not alter the colour, as far as one may judge between cloudy and clear solutions, it is believed that it merely combines with the free sulphur to form thiosulphate. At lower p_H values nothing like

full colour development is obtained in 5 minutes but it is approached with longer periods of standing. The solutions always smell of H_2S during colour development and unless plenty of reagent is used to diminish the time necessary for almost complete oxidation appreciable amounts of H_2S may be lost. At still lower p_H values the H_2S and sulphite must react in some measure to form thionic acids.

The cysteine hydrochloride used was not perfectly dry and in consequence the amounts weighed out for standard solutions were probably deficient in cysteine by 1 or 2 %. It had been prepared by reducing cystine with tin and hydrochloric acid and strict precautions were taken to prevent its re-oxidation in the course of separation. It certainly contained only the minutest trace of cystine, if any.

Now if the reagent, in being reduced by cysteine, oxidises the cysteine to cysteic acid, then, focusing attention purely upon the amounts of colour given by cysteine and by cystine *plus* sulphite, one may say that the facts do not disagree with the view that the sulphite reduces the cystine to cysteine. But if this were so, why should cysteine *plus* sulphite develop just twice the colour given by cysteine alone? The hypothesis that the sulphite reduces the cystine to cysteine and that this is oxidised by the reagent to cysteic acid, is rendered untenable by that fact alone. The second part of the hypothesis is independently invalidated by a consideration of the amounts of colour given by cysteine alone and by ferrous salt. The colour given by cysteine alone corresponds to within a few units % with the oxidation of the cysteine to cystine, not to cysteic acid. And according to this fact just established, the first part of the hypothesis must be incorrect, for if not, the colours developed by cysteine *plus* sulphite and cystine *plus* sulphite should continually increase in intensity until the sulphite is exhausted. The rôle played by cystine would then be that of catalyst to the reduction of the reagent by the sulphite, and this obviously is not the case.

The anomaly is bound up with the belief that sulphite reduces the cystine to cysteine under the conditions obtaining in the estimations. It seems that this is quite incorrect and must be abandoned. Other evidence which might appear to be against it, but is not necessarily so, is the following. Sulphite reduces the reagent only slightly, yet cysteine during its oxidation to cystine reduces strongly. Is it likely therefore that the sulphite could reduce cystine appreciably under the same conditions? The extent of reduction unfortunately cannot be calculated because the sulphite-sulphate system is irreversible under these conditions and the cysteine-cystine system is anomalous.

The following table (p. 2157) summarises the position with respect to colour development by ferrous salt, cysteine, cysteine *plus* sulphite, cystine, cystine *plus* sulphite, Na_2S, and Na_2S *plus* sulphite, the last two only in alkaline solution. The unit of colour is to be associated with 1 atom of reducing hydrogen. RSH and R—S—S—R represent cysteine and cystine respectively.

When cysteine is first carefully oxidised to cystine at p_H 5–6 by chromate,

or by H_2O_2 *plus* a trace of ferrous salt, it gives on subsequent addition of sulphite only half the colour it would have given if it had not first been oxidised.

	Amount and nature of reducer	Units of colour developed
I	$2r$ Fe^{++}	$2r$
II	$2r$ RSH	$2r$
III	$2r$ RSH + sulphite	$4r$
IV	r R—S—S—R	0
V	r R—S—S—R + sulphite	$2r$
VI	$2r$ H_2S (alkaline)	$4r$ cloudy
VII	$2r$ H_2S + sulphite (alkaline)	$4r$

The equation

$$\text{R—S—S—R} + H_2SO_3 \text{ aq.} \longrightarrow 2\text{RSH} + H_2SO_4 \text{ aq.} \quad \text{.....(2),}$$

is, as we have seen, quite unable to account for the facts.

Let it now be supposed that the action of sulphite on r molecules of cystine is to produce r molecules of cysteine *plus* s molecules of a substance X which is unable to reduce the reagent. The following formulation will suffice:

$$r\,\text{R—S—S—R} + uSO_2 + vH_2O \rightleftharpoons r\,\text{RSH} + sX \quad \text{.....(3)}^1.$$

It has also been established that:

$$2r\,\text{RSH} + \text{reagent} \rightleftharpoons r\,\text{R—S—S—R} + \text{reduced reagent} \quad \text{.....(4),}$$

the balance being found almost completely in favour of the right-hand side of the equation. The fact that the colour given by cysteine is doubled in presence of sulphite now admits of easy explanation.

Starting with equation (4) and with case II in the table, $2r$ of cysteine first give $2r$ units of colour and are converted into r of cystine. If sulphite is present then r of cystine according to equation (3) yield another r of cysteine which react with the reagent to give another r units of colour and $\frac{1}{2}r$ of cystine, and so on. The total colour developed is therefore

$$2r + r + \tfrac{1}{2}r + \tfrac{1}{4}r + \tfrac{1}{8}r + \text{.....}$$

which, in the limit, becomes $2r + 2r$ or $4r$.

The colour developed by r of cystine alone is 0, and by r of cystine *plus* sulphite is $2r$. The case admits of similar explanation to that given above. The initial $2r$ of colour is of course missing and the total colour developed is merely that of the summation, $r + \frac{r}{2} + \frac{r}{4} + \textit{etc.}$, becoming $2r$ in the limit.

Thus equations (3) and (4) account satisfactorily for the known facts. The reaction expressed by (4) is quite rapid over the p_H range 5–8·4, the greatest rate of colour development occurring in the most alkaline solutions. From the very low rate of colour development with cystine *plus* sulphite at low p_H values compared with the rate at higher values, it has been concluded that (*a*) the reaction velocities in equation (3) are very small in the acid region,

[1] The double reaction arrows in this and following equations must be taken only to represent a possible equilibrium of some sort, not necessarily of the type such equations ordinarily represent.

or (*b*) the velocities may be high but with equilibrium well in favour of the left-hand side in acid solutions, or (*c*) both (*a*) and (*b*) might apply.

From the point of view of interest in the *X* compound of equation (3), the following considerations and experiments are appended.

The simplest form equation (3) may take is

$$R—S—S—R + SO_2 + H_2O \rightleftharpoons RSH + RHS_2O_3 \quad \text{.....(5),}$$

in which *sX* becomes RHS_2O_3.

Equation (5) represents a reaction somewhat similar to that given for the action of cyanides upon cystine

$$R—S—S—R + NaCN \rightleftharpoons RSNa + RCNS \quad \text{.....(6).}$$

An alternative to equation (5) as a particular case of equation (3) would be

$$3R—S—S—R + 8SO_2 + 5H_2O \rightleftharpoons 3RSH + 2H_2S_4O_6 + 3RSO_3H \quad \text{...(7)}$$

in which the *X* compound would occur as a mixture of tetrathionic and cysteic acids, both of which are fairly stable and not reactive toward the reagent. On the evidence thus far submitted equations (5) and (7) would perhaps have equal claims to being the correct one.

Organic thiosulphates of the form postulated in equation (5) are by no means unknown. Purgotti [1892], investigating the thiosulphonyl derivative of glycollic acid, prepared it by treating chloroacetic acid with sodium thiosulphate. Aqueous solutions of the salts are not readily decomposed by boiling, but, in contrast to the action of mineral acids upon inorganic thiosulphates, boiling hydrochloric acid converts the barium salt of the thiosulphonyl derivative of glycollic acid, for example, into barium sulphate and thioglycollic acid.

It was found that a stream of SO_2 could be blown into a solution of cystine in excess of hydrochloric acid without any chemical change occurring. No sulphate was produced and none of the cystine was affected in any way. On boiling to expel SO_2 the solution was found to contain the original cystine and hydrochloric acid and nothing else. The original cystine could be accounted for colorimetrically and the total sulphur content corresponded with the amount of cystine present.

A mixture containing cystine and $NaHSO_3$ was made alkaline at p_H 12–13, then acid, alkaline again, and acid again, all in the cold. No sulphate was produced. An aliquot was allowed to develop its colour with the reagent in presence of more sulphite and the colour was compared with that of a standard cystine + sulphite solution containing the same amount of cystine as that originally present in the aliquot. There was a 7 % loss of colour. Another aliquot was boiled to expel SO_2 and then allowed to reduce the reagent in presence of sulphite. There was a gain in colour developed of 77 %. In Part II of this series, methods for estimating cysteine and cystine will be discussed in detail. Applying them to analysis of the boiled aliquot it was found that

81 % of the cystine had been converted into cysteine, 13 % remained unchanged, and the balance, 6 %, had been lost to colorimetric estimation. Gravimetric analyses of sulphate and total sulphur confirmed these results and revealed that the sulphur of the 6 % cystine lost to colorimetry remained in the solution, probably as cysteic acid.

These experiments throw a little light on the nature of the *X* compound which is certainly elusive but not necessarily chimerical. If the thiosulphonyl derivative of α-aminopropionic acid is the *X* compound and reacts with boiling mineral acid as does Purgotti's thiosulphonyl derivative of glycollic acid, the observations are automatically explained. There can really be little doubt that equation (5) is the correct form of equation (3).

Summary.

1. The reduction of solutions of phospho-18-tungstic acid by different types of substances has been investigated colorimetrically under varying conditions, especially in respect to varying p_H. Some attempt has been made to describe the results in general terms of reaction velocities and equilibria. Reductions by cysteine have been examined in some detail.

2. Evidence has been adduced to show that the reaction between cystine and sulphites in cold aqueous solution is not a "reduction" in the ordinary sense of the term, but that it is most probably expressed by the equation, $R—S—S—R + H_2SO_3 \rightleftharpoons RSH + RHS_2O_3$, where R—S—S—R and RSH represent cystine and cysteine respectively.

I wish to express my indebtedness to Sir Charles J. Martin for his interest in this work and for correcting the manuscript and arranging for its publication.

References.

Andrews (1928). *J. Biol. Chem.* **80**, 191.
Folin and Denis (1912). *J. Biol. Chem.* **12**, 239.
—— and Looney (1922). *J. Biol. Chem.* **51**, 421.
—— and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
—— and Trimble (1924). *J. Biol. Chem.* **60**, 473.
Heffter (1907). *Chem. Zentr.* **2**, 822.
Hunter and Eagles (1927). *J. Biol. Chem.* **72**, 177.
Kehrmann (1887). *Ber. deutsch. chem. Ges.* **20**, 1805.
Michaelis and Flexner (1928). *J. Biol. Chem.* **79**, 689.
Purgotti (1892). *Gazzetta*, **22**, 422.
Rimington (1930). *Biochem. J.* **24**, 1114.
Sullivan (1926). *U.S. Pub. Health Serv. Reports*, **41**, 1030.
Tompsett (1931). *Biochem. J.* **25**, 2014.
Wu (1920). *J. Biol. Chem.* **43**, 189.

CCLIV. THE APPLICATION OF PHOSPHO-18-TUNGSTIC ACID (FOLIN'S REAGENT) TO THE COLORIMETRIC DETERMINATION OF CYSTEINE, CYSTINE AND RELATED SUBSTANCES.

II. THE DETERMINATION OF SULPHYDRYL COMPOUNDS AND DISULPHIDES ALREADY EXISTING IN SOLUTION.

By JOSEPH W. H. LUGG.

From the Division of Animal Nutrition of the Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia.

(*Received September 12th, 1932.*)

The principles underlying the new methods will be indicated briefly, but to form an idea of the magnitudes and likelihood of errors and for a proper understanding of the principles involved, it will be necessary to refer to Part I of the series [1932].

By estimating cysteine and cystine in acid solution all the disadvantages of alkaline colour development may be avoided. The disadvantages of acid colour development include slight loss in colour and diminution in rate of development. For all round utility a p_H of 5·7 has been selected, and to minimise colour development by extraneous reducers and for the sake of economy, only small amounts of reagent are employed. A cystine *plus* sulphite standard is used for comparison.

The cysteine is allowed to develop its coloration in absence of sulphite and its estimation in absence of extraneous reducers is quite simple. In coloration developed, 1 mol. of cystine = 2 mol. of cysteine, or 2 mg. of cystine = 2·017 mg. of cysteine. When extraneous reducers are present their effects must be found by artifice. In the presence of mercuric chloride cysteine gives no coloration at all, and ferrous salt is used to find the contribution of the extraneous reducers to the total coloration developed. Since $HgCl_2$ will form a precipitate with uric acid, and because it influences slightly the coloration developed by some extraneous reducers, it is necessary to have a fairly large amount of zinc chloride present to prevent precipitation and to provide an initial "heavy metal" effect upon the weaker reducers so that the effect of the $HgCl_2$ will be diminished. To nullify the inhibiting effect of the $ZnCl_2$ upon the rate of colour

development by cysteine, it is desirable that some NH_4Cl be present also. The $ZnCl_2$ and NH_4Cl are incorporated with the citrate buffer used in preparing standard and unknowns.

Cystine is estimated with sulphite present and the estimation is simple when extraneous reducers are absent. The same buffer as is used in estimating cysteine is employed. When extraneous reducers are present, mercuric chloride and ferrous salt are used as in the corresponding cysteine estimation.

Provided extraneous reducers are absent, the estimations of cysteine and cystine together are not difficult. In absence of sulphite the coloration is due to cysteine alone. The cystine develops its usual colour in presence of sulphite and the cysteine develops twice as deep a coloration and hence, subtracting twice the value obtained in absence of sulphite from that obtained in presence of sulphite, the nett amount represents the cystine. In so far as the cysteine in the unknown solution may be converted fairly rapidly into cystine by aerial oxidation, it might be preferable to record the "total cystine" (cysteine *plus* cystine) content by subtracting the value of the coloration obtained in absence of sulphite from that obtained in its presence and calculating the nett amount on the basis of 2 mg. of cystine = 2 mg. of "total cystine." The solution which develops its coloration in presence of sulphite should develop most of its cysteine coloration in absence of the sulphite, which is added after a short interval. By this precautionary measure the "total cystine" might be estimated quite accurately even if a considerable amount of the cysteine were to suffer aerial oxidation during colour development. Provision is made for this in the method, but tests so far have shown that the precaution is not necessary.

When extraneous reducers are present they may contribute different amounts of colour in absence and presence of sulphite. Mercuric chloride and ferrous salt are used in absence and presence of sulphite to find the two contributions.

The use of mercuric chloride renders these methods highly specific for sulphydryl compounds and disulphides, and in analysing any solution it is wise to assume that extraneous reducers are present and to proceed accordingly.

Neither cysteine nor cystine is extracted from acid aqueous solution (p_H less than 3) by ether, but part of the cysteine may be oxidised to cystine in the process of extracting a solution. On the other hand laevulic acid, pyruvic acid, furfuraldehyde, and similar substances may all be extracted though never completely. Their contributions to the coloration developed may thus be greatly reduced and the possible error in a cystine estimation thereby diminished. Four extractions with twice the volume of ether each time will remove approximately four-fifths of the laevulic and pyruvic acids and practically all the furfuraldehyde. Ether extractions introduce a decided degree of specificity for cysteine and cystine among the sulphydryl compounds and disulphides. The condition that these compounds should remain in the aqueous acid solution is probably that they contain a strongly basic group like $—NH_2$.

Complications arise when the unknown solution is coloured initially. Selective absorbents should never be used as their selectivity can never be perfect and is frequently very poor. Theoretically the compensating colorimeter is the instrument to be employed and the blank used with the standard must or must not contain sulphite according to its presence or absence in the unknown. There is always some uncertainty in the matter of the blanks because one cannot know what effect the reagent would have upon the adventitious coloration.

If the adventitious coloration is the same as that being developed its value may be subtracted from the readings obtained when an ordinary colorimeter is employed. Even when the adventitious coloration is at the other end of the spectrum, as in protein hydrolysates, an experienced operator may blind himself to it, if it is not extremely pronounced, and achieve excellent results with an ordinary colorimeter. It is the author's experience that most compensating instruments fail to survive even those simple tests to which an ordinary instrument is put before being used.

The reagents, which are listed below, should be filtered through sintered glass if necessary. The list is followed by directions for making estimations.

Phospho-18-tungstic acid reagent. This is prepared according to the directions of Folin and Marenzi [1929] with separation of any molybdenum present according to their plan. It may be looked upon as containing per litre 100 g. of the salt $Na_2WO_4, 2H_2O$, 172 g. of H_3PO_4, and 16·1 g. of LiOH. The sodium tungstate should be prepared by dissolving the necessary amount of precipitated WO_3 in the necessary amount of NaOH solution. If the reagent fails to comply with the following requirements it must be discarded. (1) It must give no coloration with tyrosine or tryptophan at any p_H. (2) At p_H 5·7 and 8·4, 4 mg. of cystine in presence of 2 cc. of reagent and 1 millimol. of sulphite must give not less than 3·95 times the coloration developed by 1 mg. of cystine. (3) At p_H 5·7 and 8·4 and in absence of zinc and ammonium salts, 80 mg. of cysteine with 2 cc. of reagent must give almost exactly seven times the coloration developed by 2 mg. of cysteine.

Sodium acetate solution: a 4 *M* solution containing 544 g. of the salt $C_2H_3O_2Na, 3H_2O$ per litre.

Composite citrate buffer. This contains 105 g. of crystalline citric acid $C_6H_8O_7, H_2O$, 52·5 g. of NaOH, 13·6 g. of $ZnCl_2$ and 26·8 g. of NH_4Cl per litre. It is best prepared by shaking the citric acid and NaOH with about 500 cc. of water with constant cooling to avoid boiling, until entirely dissolved. The $ZnCl_2$ is dissolved in 20 cc. of water and added to the cold solution, and this is followed by the NH_4Cl dissolved in about 200 cc. of water. The mixture is diluted to 1 litre.

Buffered sulphite. 9·5 g. of sodium metabisulphite ($Na_2S_2O_5$) are dissolved in about 70 cc. of water, 15 cc. of 4 *M* sodium acetate solution are added and the mixture is diluted to 100 cc. This *M* sulphite solution will keep for months in a stoppered bottle.

Mercuric chloride: a 0·1 *M* solution containing 2·72 g. of $HgCl_2$ per 100 cc. of solution.

Ferrous salt: a *M*/12 Fe^{++} solution containing 3·268 g. of Mohr's salt ($FeSO_4$, $(NH_4)_2SO_4$, $6H_2O$) and 8·5 cc. of *N* H_2SO_4 per 100 cc. It will keep for months in a well-stoppered bottle and may be titrated against potassium permanganate to check alteration. The solution is diluted 1 in 8, 1 in 9, 1 in 10, *etc.*, before use in the estimations.

Standard cystine solution. 0·5 g. of pure cystine (*l*-cystine is most easily obtained) is dissolved in 50 cc. of *N* hydrochloric acid, and diluted to 500 cc. to provide a *M*/240 solution in 0·1 *N* HCl, containing 1 mg. of cystine per cc. The solution will keep indefinitely.

Estimations in general.

All estimations are carried out in standard 100 cc. flasks. Standard and unknown should be of approximately the same colour intensity (70–150 %) and must be prepared together. In reference to the 5–10 minute interval allowed for colour development, one of about 7 minutes is to be preferred. The solutions should be read within 30 minutes of being diluted to the mark.

The standard cystine solution is acid and every 1 cc. requires 0·25 cc. of the sodium acetate solution to be brought to p_H 5·7. Aliquots of the unknown cysteine, cystine, and mixed solutions must be titrated to p_H 5·7 with the sodium acetate solution, using bromocresol purple indicator. The appropriate amounts of acetate must be included with the larger amounts mentioned in the schedules below when preparing the standards and unknowns.

Estimation of cysteine.

Unknown. 10 cc. of citrate buffer, 2 cc. of reagent and 2 cc. of sodium acetate solution are mixed in a flask in that order, and to the mixture is added the chosen aliquot of unknown cysteine solution.

Standard. To a mixture of the same composition as that used for the unknown is added the necessary amount of cystine solution and this is followed by 1 cc. of buffered sulphite.

After 5–10 minutes the solutions are diluted and compared. The apparent cysteine content will be $x + a$ mg., where x is the true amount; in absence of extraneous reducers a will be zero. If extraneous reducers are present a is found as follows. Another unknown is prepared just as before, but, prior to adding the unknown cysteine solution, 1 cc. of the mercuric chloride solution is added to the mixture. The amount of coloration developed, calculated as cysteine, will correspond with A mg. By trial and error in a series of solutions, the amount of ferrous salt necessary to make the total coloration agree closely with the original apparent $x + a$ mg. of cysteine, is found. A new unknown is prepared containing the mercuric chloride and unknown cysteine solutions followed by the necessary amount of ferrous salt, and compared with a new

standard containing precisely the same amount of ferrous salt but none of the unknown cysteine solution. The contribution of the extraneous reducers to the colour developed by the ferrous salt is found to be a_1 in terms of mg. of cysteine. a_1 may be different from A but it is nearly the same as a, and hence x is found from $x + a - a_1$.

Estimation of cystine.

The standard and unknown are prepared in precisely the same manner as the standard cystine *plus* sulphite solution used in estimating cysteine. The apparent amount of cystine found will be $y + b$ mg., where y is the true amount, and b is zero when extraneous reducers are absent. If extraneous reducers are present then b is found as follows. Another unknown is prepared just as before and containing sulphite, but prior to adding the unknown cystine solution 1 cc. of the mercuric chloride solution is introduced. The coloration will correspond with b mg. of cystine. By trial and error the amount of ferrous salt necessary to make the total coloration agree closely with the original apparent $y + b$ mg. of cystine is found. A new unknown is prepared containing mercuric chloride, the unknown cystine solution, and sulphite, and after colour development has proceeded for about 1 minute the necessary amount of ferrous salt is added. Colour development is allowed to continue for the remainder of the 5–10 minutes and on dilution the solution is compared with a new standard containing mercuric chloride, sulphite, and the same amount of ferrous salt, but none of the unknown cystine solution. The contribution of the extraneous reducers to the colour developed by the ferrous salt in presence of sulphite is thus found to correspond with b_1 mg. of cystine. Identifying b_1 with b, y is found from $y + b - b_1$.

Estimation of cysteine and cystine when present together.

The procedure is merely a combination of those used in estimating cysteine and cystine. Suppose there are x mg. of cysteine and y mg. of cystine present in a chosen aliquot. In absence of extraneous reducers the colour developed without sulphite will correspond with the x mg. of cysteine. With sulphite the colour will correspond with $2x$ mg. of cysteine *plus* y mg. of cystine. Hence both x and y are found. If extraneous reducers are present the coloration in absence of sulphite will correspond with $x + a$ mg. of cysteine, and in presence of sulphite it will correspond with $2x$ mg. of cysteine *plus* $y + b$ mg. of cystine. In absence of sulphite mercuric chloride is used first to find a and later with ferrous salt to find a_1. In presence of sulphite it is used first to find b and then with ferrous salt to find b_1. a_1 is assumed identical with a, and b_1 with b, and hence both x and y may be found.

None of the coloured solutions used in estimating cysteine, cystine, and mixtures of the two, should exceed in intensity the coloration developed by 4 mg. of cystine in presence of sulphite.

SUMMARY.

Colorimetric methods involving the use of phospho-18-tungstic acid (Folin's reagent) are described for the estimation of sulphydryl compounds and disulphides, with especial reference to cysteine and cystine.

The estimations may be performed in the presence of large amounts of extraneous reducers. Under such conditions the methods are rather tedious but have been found to yield reasonably accurate results.

The author wishes to express his indebtedness to Sir Charles J. Martin for continued interest and assistance in publication.

REFERENCES.

Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Lugg (1932). *Biochem. J.* **26**, 2144.

CCLV. THE RECIPROCAL RELATIONSHIP OF CALCIUM AND INORGANIC PHOSPHORUS OF THE BLOOD OF SHEEP.

By ALLAN HENRY HECTOR FRASER.

From the Rowett Research Institute, Aberdeen.

(*Received May 14th, 1932.*)

Howland and Kramer [1923] showed that in rickets the concentration product of calcium and inorganic phosphorus falls below a certain critical level. Their work suggested a fundamental relationship between the levels of Ca and inorganic P of the blood.

Bourne and Campbell [1932] by simultaneous determinations of calcium and inorganic P of the blood in rabbits have shown that the concentrations of these two substances bear a reciprocal relation to each other. Their work is fully supported by the work of Dupré and Semeonoff [1931]. On the other hand Theiler, Green and du Toit [1927] found in their extensive work on cattle that the blood-Ca remained normal even with an inorganic P of one quarter the normal figure. This contradiction might suggest that in this respect ruminants differed fundamentally from rabbits.

Recent work by Auchinachie and Fraser [1932], however, on the blood of sheep fed a Ca-deficient ration has shown that an abnormally low serum-Ca may be accompanied by an abnormally high inorganic P, and that there is evidence of a reciprocal relationship between the Ca and inorganic P of the blood of sheep.

Methods.

In November 1930 five sheep were put on a Ca-deficient diet. Five other sheep were put on the same diet *plus* 5 cc. of cod-liver oil per sheep per day. Simultaneous estimations of blood-Ca and inorganic P were made at intervals in each individual of both groups. Both groups were kept out of doors.

Results.

The average figures for serum-Ca are given in Table I. In Group I the serum-Ca fell to the low level of 7·06 mg./100 cc. in March rising to 8·15 in June. During the same period (November–June) the addition of cod-liver oil maintained the serum-Ca at a normal level.

Table I. *Ca in mg./100 cc.*

	January	March	June
Group I (Ca-deficient)	10·65	7·06	8·15
Group II (Ca-deficient + cod-liver oil)	11·10	10·25	10·00

The average figures for inorganic P are given in Table II. It is apparent that while the P in both groups shows a seasonal increase, the blood-P in the Ca-deficient group was very much higher than when cod-liver oil was fed, particularly in March.

Table II. *P in mg./100 cc.*

	January	March	June
Group I	5·63	10·53	9·15
Group II	6·11	7·29	7·66

It is evident that there is a significant difference between the two groups both in blood-Ca and blood-P.

There is, however, a remarkably close correspondence between the Ca and P concentration products of the two groups, as is shown by Table III.

Table III. *Ca × P concentration product.*

	January	March	June
Group I	67·1	72·3	77·3
Group II	67·8	74·7	76·3

In each group there is evidence of a seasonal rise in the product, but at any one period there is a close correspondence between the products of both groups. This is important in view of the divergent values obtained for Ca alone and for P alone. It suggests that the Ca and P levels of the blood in sheep are reciprocally related, the P level rising proportionately to the fall in Ca and *vice versa*, a result in keeping with the results of Bourne and Campbell and of Dupré and Semeonoff on rabbits.

Discussion.

These results on sheep were obtained on small groups and have not yet been repeated. They must therefore be regarded as of a preliminary nature. At the same time the evidence of a reciprocal relationship in this particular case is perfectly clear and is in agreement with similar results on rabbits. In discussing the possible interpretation of this relationship, there are several main possibilities to consider. In the first place the basal diet whilst deficient in Ca was adequate in P. It is conceivable that the blood-P might rise owing to the Ca-deficiency leading to a reduced rate of formation of insoluble compounds of Ca and P in the intestine. This interpretation is ruled out by the effect of cod-liver oil, where no extra lime entered the intestines, and yet the blood-P fell markedly below that of the Ca-deficient group.

It seems more probable that the phenomena of reciprocal relationship of

the Ca and P of the blood are bound up with the metabolism of bone in the manner suggested in the following hypothesis.

As a result of deficiency in the food, the amount of Ca absorbed from the gastro-intestinal tract is insufficient to maintain the normal level of serum-Ca, and the calcium reserves of the bones are utilised to supply the needs of more essential tissues. But since Ca is present in bone mainly as calcium phosphate the transference of calcium from bone to blood will be accompanied by a simultaneous transference of phosphorus. This view is supported by the work of Evans [1930] on swine, and of Bekker and Rousseau [1930] on sheep, which shows that neither a Ca nor a P deficiency in the diet alters the percentage composition of the bones. Provided that P is being assimilated in normal amount, the liberation of bone phosphate will lead to a rise in the blood-P level, and the extent of this rise will be proportionate to the rapidity of mobilisation of the calcium reserves in the bones. Cod-liver oil by raising the serum-Ca level, reduces the demand on the Ca bone reserves, decreases the liberation of bone phosphate, and hence lowers the blood-P level. On this hypothesis the blood-P will rise in response to a fall in serum-Ca, and fall in response to a serum-Ca rise, thus leading to a reciprocal relationship of Ca and P in the blood, and to a relative constancy of the Ca $\times$ P concentration product.

In cases where both calcium and phosphorus are deficient in the diet, or where conditions favouring assimilation are sub-normal, it is obvious that this reciprocal relationship will not be clearly shown.

Summary.

1. In sheep under outdoor conditions, fed a basal ration deficient in calcium, or such a ration supplemented with cod-liver oil, the Ca $\times$ P product of Howland and Kramer tends to approach a constant, in spite of wide variations in serum-calcium and blood-inorganic phosphorus produced by differences in diet.

2. The relative constancy of the Ca $\times$ P product is shown to depend upon the reciprocal relationship of the calcium and phosphorus of the blood, the level of phosphorus rising as that of calcium falls, and *vice versa*.

3. It is suggested that this reciprocal relationship is due to factors involved in utilisation of the mineral reserves of the bones.

REFERENCES.

Auchinachie and Fraser (1932). *J. Agric. Sci.* **22**, 560.
Bekker and Rousseau (1930). *16th Rep. of Director Vet. Services and Animal Industry, Onderstepoort*, p. 293.
Bourne and Campbell (1932). *Biochem. J.* **26**, 183.
Dupré and Semeonoff (1931). *J. Biol. Chem.* **94**, 341.
Evans (1930). *J. Agric. Sci.* **20**, 117.
Howland and Kramer (1923). *Monatschr. Kinderheilk.* **25**, 279.
Theiler, Green and du Toit (1927). *J. Agric. Sci.* **17**, 291.

CCLVI. THE RELATION OF THIOL COMPOUNDS TO GLUCOSE FERMENTATION.

By JUDA HIRSCH QUASTEL
and ARNOLD HERBERT MAURICE WHEATLEY.
From the Biochemical Laboratory, Cardiff City Mental Hospital.

(*Received November 1st, 1932.*)

In the course of experiments on the toxicity of iodoacetic acid to biological processes, it was found that addition to the acid of sodium thiosulphate or thiol compounds brought about a considerable diminution in the intensity of the toxic effect. This observation led the writers to consider the possibility that thiol compounds may normally play an important part in the processes affected by iodoacetic acid. Experiments were accordingly devised to test this possibility, especially with regard to yeast fermentation. The results which are recorded below showed that both cysteine and glutathione markedly affect the relationship of aerobic fermentation of glucose by baker's yeast to the respiration in presence of glucose. It would seem that glutathione plays some controlling part in determining the relationship between aerobic fermentation and respiration of the cell in presence of glucose.

A. Experiments with iodoacetic acid.

Experiments with animals showed that the toxicity of iodoacetic acid was greatly diminished by admixture with sodium thiosulphate. For instance, an intramuscular injection of 3 cc. of a 2 % solution of iodoacetic acid (neutralised with NaOH) was invariably fatal to guinea-pigs, death occurring within 1 hour after the injection. When, however, 0·3 g. sodium thiosulphate was dissolved in this dose of iodoacetic acid death of the animal did not occur. A separate injection of thiosulphate did not relieve the animal from the toxic action of iodoacetic acid and it was apparent that the diminution of toxicity of this acid by the presence of thiosulphate was due to interaction between the two substances.

This was confirmed by iodimetric titration of sodium thiosulphate solution after the addition of iodoacetic acid. Two solutions were prepared (*a*) 50 cc. $N/100$ sodium thiosulphate, (*b*) 50 cc. $N/100$ sodium thiosulphate to which was added sodium iodoacetate to give a concentration of $N/250$. The solutions were placed at 37° for 3 hours after which titrations on 5 cc. with $N/100$ iodine solution were made. The titrations were (*a*) 5·0 cc., (*b*) 3·15 cc. The theoretical

figure for (*b*) was 3·0 cc. had there been complete interaction between one molecule of sodium thiosulphate and one molecule of iodoacetic acid.

Experiments were then carried out to determine whether the halogen atom of iodoacetic acid was sufficiently labile to react with the thiol groups of cysteine and glutathione.

In performing these experiments, advantage was taken of the fact that exposure of brain tissue to dilute iodoacetic acid resulted in an inability of the tissue to oxidise glucose and other sugars [Krebs, 1931; Quastel and Wheatley, 1932].

Fresh brain tissue was added to solutions containing (*a*) iodoacetic acid, (*b*) iodoacetic acid + cysteine, (*c*) iodoacetic acid + glutathione, (*d*) iodoacetic acid + sodium thiosulphate. It was found that the inhibitory action of iodoacetic acid on the oxidation of glucose by brain was much reduced by its previous admixture with thiol compounds or sodium thiosulphate.

The experimental technique, which involved the use of the Barcroft differential manometer, was that fully described in an earlier communication [Quastel and Wheatley, 1932]. Briefly it consisted in placing in the right-hand vessel of the manometer 1 cc. *M*/5 phosphate buffer solution p_H 7·4, 2 cc. saline (containing the iodoacetic acid or mixture of iodoacetic acid and sulphur compound) and 0·5 g. fresh chopped guinea-pig brain tissue. 3 cc. saline were placed in the left-hand vessel. The apparatus was shaken at the temperature of the bath (37°) for 2½ hours when the rate of autoxidation of the brain had fallen very considerably. Glucose was then added to the tissue to give a final concentration of 0·025 % and the new rate of oxygen uptake was observed. This was compared with the rate given by brain tissue which had not been treated with iodoacetic acid. The p_H of all solutions added to the brain tissue was 7·4—acids being neutralised with sodium hydroxide. The results are shown in Table I.

Table I.

Oxygen uptakes in mm.³ by 0·5 g. guinea-pig brain tissue (in presence of 0·025 % glucose) after treatment with iodoacetic acid and mixtures of iodoacetic acid and sulphur compounds. Autoxidation had been allowed to proceed for 2½ hours before addition of the glucose.

Mode of treatment	Time (in mins.) 30	60	90	120
Control (no iodoacetic acid)	144	312	474	618
M/4000 iodoacetic acid	52	95	124	150
,, + *M*/160 cysteine	108	235	356	482
,, + *M*/100 glutathione	128	270	408	548
,, + 0·7 % sodium thiosulphate	92	187	285	382
Cysteine *M*/160	124	266	404	538
Glutathione *M*/100	142	302	460	612
Sodium thiosulphate (0·7 %)	147	313	471	610

It will be observed that cysteine, glutathione and sodium thiosulphate diminish the toxic action of iodoacetic acid, a considerably greater rate of oxygen uptake being secured. Control experiments showed that none of those

sulphur compounds, when added alone, appreciably affected the oxygen uptake of brain tissue in the presence of glucose. Cysteine had the most marked action.

Experiments were then carried out to determine whether the addition of cysteine *etc.* to brain tissue already exposed to iodoacetic acid for 2½ hours, would restore the normal rate of O_2 uptake in the presence of glucose. The results were negative showing that the alleviating action of the sulphur compounds when added together with the iodoacetic acid was due to direct interaction with the halogen acid.

Some evidence for the interaction of thioglycollic acid with iodoacetic acid has been obtained by Bersin [1932].

These experiments demonstrate the lability of the halogen atom of iodoacetic acid. The comparative ease with which it combines with thiol groups would lead one to consider the possibility that the physiological activity of iodoacetic acid may be connected with an interaction with naturally occurring thiol groups in the living cell. If this were so, it would be anticipated that thiol compounds may play a part in some of the biochemical reactions specifically affected by iodoacetic acid.

In view of the fact that fermentation of glucose by yeast is inhibited by small concentrations of iodoacetic acid [*cf.* Lundsgaard, 1932] experiments were undertaken to determine whether thiol compounds play any rôle in the process of glucose fermentation by yeast.

B. Experiments with yeast.

Technique.

In the experiments to be described the Barcroft differential manometer was used. Observations were made of the O_2 uptake and CO_2 output by yeast in the presence of phosphate buffer solution of p_H 6·0 containing 1·6 % glucose. Two manometers were used for the estimations. In the first manometer 0·3 cc. 20 % KOH was placed in the inner tube of the reaction vessel, the O_2 uptake alone of the yeast being obtained. In the second manometer potash was omitted from the reaction vessel, the readings obtained representing differences between CO_2 output and O_2 uptake. The CO_2 output was determined from the readings of the two manometers. It is important in this work that the p_H of the fluids in the vessels of the manometers should remain the same throughout the experimental period. Using $M/15$ phosphate buffer of p_H 6·0 it was found that no appreciable difference in p_H occurred.

The effect of a thiol compound on the metabolism of yeast was obtained either by adding the substance to the yeast at the commencement of an experiment or by adding it to the yeast after fermentation had proceeded for some time. Actually it was found that the effects of the thiol compound were the same by either method.

Experiments were carried out at 37°, air being present in the Barcroft

manometer vessels. Readings were taken after preliminary shaking for 15–30 minutes to ensure temperature equilibrium and steady rates of O_2 uptake and CO_2 output.

Most of the work so far has been carried out with fresh baker's yeasts—two Belgian varieties and one Dutch (Delft) being used.

The exact procedure was as follows.

In the right-hand vessels A, B, C, D of four Barcroft differential manometers were placed:

(A) 1 cc. $M/5$ phosphate buffer solution p_H 6·0, 1 cc. saline, 0·5 cc. 10 % glucose solution (in saline), 0·5 cc. 2 % yeast suspension (in saline); no potash was present;

(B) as in (A), but potash was placed in the inner tube;

(C) as in (A), with the exception that 1 cc. saline was replaced by 0·5 cc. saline and 0·5 cc. of 0·5 % solution of thiol compound (in saline); no potash was present;

(D) as in (C), but potash was placed in the inner tube.

The left-hand vessels each contained 3 cc. saline. Readings were taken every 15 minutes over a period of 3 hours. At the close of the experiment p_H estimations were made to determine whether material changes of p_H had occurred. As a rule no difference in p_H greater than 0·2 was observed.

Results with cysteine.

The rate of CO_2 evolution is slightly greater than the rate of O_2 uptake with all specimens of baker's yeast used, under the experimental conditions described.

The addition of cysteine to the yeast entirely changes the rates of CO_2 output and O_2 uptake. There is an enhanced rate of production of CO_2 and a greatly diminished rate of O_2 uptake.

The results in Table II are typical of cysteine (a Belgian yeast being used). 2·5 mg. cysteine hydrochloride (neutralised with NaOH) were present.

Table II.

		Time (in mins.)			
		30	60	90	150
No cysteine present.	O_2 uptake, mm.³	290	513	753	1204
	CO_2 output, mm.³	334	582	847	1355
Cysteine present.	O_2 uptake, mm.³	79	148	209	308
	CO_2 output, mm.³	414	762	1078	1664

Similar results were obtained with Delft baker's yeast. It is evident that cysteine exercises a considerable inhibitory action on the O_2 uptake of yeast in the presence of glucose. This result is quite different from that obtained with brain tissue where cysteine has relatively little inhibitory action on the oxygen uptake in the presence of glucose (Table I).

The effect of cysteine on the O_2 uptake and CO_2 output resembles that of cyanide, with the exception that the inhibitory effect of cyanide on yeast respiration is much more pronounced.

The increase in the rate of CO_2 formation by the cysteine only occurs under aerobic conditions. Under anaerobic conditions or in the presence of cyanide cysteine does not increase the rate of CO_2 formation—it has on the contrary a small inhibitory action. Thus, in one experiment, in the presence of 0·015 % HCN the evolution of CO_2, under the conditions given above (and using a Belgian yeast), was 503 mm.^{3} in 60 mins. and 930 mm.^{3} in 120 mins. In the presence of 0·015 % HCN and 0·15 % cysteine the evolution of gas was 459 mm.^{3} in 60 mins., and 821 mm.^{3} in 120 mins., indicating some toxicity of cysteine to the fermenting complex.

Results with glutathione.

The action of glutathione is similar to that of cysteine in bringing about a considerable change in the relative rates of CO_2 output and O_2 uptake. Whereas, however, cysteine greatly diminishes the O_2 uptake of yeast in the presence of glucose, glutathione has a relatively small effect.

The following results (Table III) are typical for glutathione (a Delft yeast being used).

Table III.

		Time (in mins.)		
		30	60	90
No glutathione added.	O_2 uptake, mm.^{3}	314	577	833
	CO_2 output, mm.^{3}	414	761	1120
2·5 mg. glutathione added.	O_2 uptake, mm.^{3}	293	512	702
	CO_2 output, mm.^{3}	534	1149	1784

Glutathione brings about a gradual decrease in the rate of O_2 uptake and a decided increase in the rate of CO_2 output. Moreover the rate of CO_2 output appears to increase with time in presence of added glutathione.

Preliminary experiments have indicated that the greater the quantity of glutathione added, the greater is the rate of evolution of CO_2, the limiting rate being equal to that obtained when glucose is fermented under anaerobic conditions (*i.e.* in nitrogen and in the absence of added SH compounds[1]). Thus the ratios of the rates of formation of CO_2 with baker's yeast in the presence of 1·6 % glucose in nitrogen, in air and in the presence of $M/400$ glutathione and air were 100 : 60 : 94.

Effect of cysteine on fermentation of fructose.

Cysteine behaves in the same way towards the fermentation of fructose as towards that of glucose.

The following results (Table IV) were obtained using 0·5 cc. of a 5 % suspension of a baker's yeast, the conditions being those described for glucose. 2·5 mg. cysteine hydrochloride (neutral) were added.

[1] The effect of glutathione varies quantitatively with the type of yeast. The tripeptide was found to enhance the rate of CO_2 formation with a Delft baker's yeast at concentrations which were ineffective with a Belgian yeast.

Table IV.

		Time (in mins.)			
		30	60	90	120
No cysteine present.	O_2 uptake, mm.^{3}	547	1055	1532	1942
	CO_2 output, mm.^{3}	597	1189	1837	2499
Cysteine present.	O_2 uptake, mm.^{3}	77	134	189	211
	CO_2 output, mm.^{3}	864	1726	2584	3341

Effect of cysteine on respiration of yeast in presence of glycerol.

The action of cysteine on the respiration of yeast in the presence of glycerol (0·5 cc. of 10 % solution) was investigated. The following results (Table V) were obtained, using 0·5 cc. of a 5 % suspension of baker's yeast.

Table V.

		Time (in mins.)		
		30	60	90
Cysteine absent.	O_2 uptake, mm.^{3}	150	279	391
	CO_2 output, mm.^{3}	100	191	273
4 mg. cysteine added.	O_2 uptake, mm.^{3}	133	244	336
	CO_2 output, mm.^{3}	113	214	298

It will be observed that cysteine had a relatively small effect either on the CO_2 output or on the O_2 uptake of yeast in the presence of glycerol.

It is evident from these results with glycerol and from the lack of a marked inhibition of O_2 uptake by brain in the presence of glucose that the inhibitory effect of cysteine on hexose oxidation by yeast is intimately associated with a specific process occurring in the breakdown of the hexose molecule by yeast.

Mode of behaviour of cysteine and glutathione.

It is apparent from the results cited that thiol compounds as represented by cysteine and glutathione affect markedly the relationship between the oxidation and the fermentation of glucose by yeast. The liberation of CO_2 approaches the rate obtained when yeast ferments glucose under anaerobic conditions. Whereas, however, the O_2 uptake by yeast in presence of glucose is greatly inhibited by cysteine, glutathione has but a small effect. The phenomena observed with these thiol compounds are similar to those described by Warburg [1926] in his studies of the relative effects of cyanide and carbylamine on the Pasteur reaction. Carbylamine intensified aerobic fermentation without any marked action on the respiration, whilst cyanide greatly decreased the respiration.

As Warburg has pointed out, many substances, *e.g.* narcotics, will affect the Pasteur reaction, the normal relationship between aerobic fermentation and respiration being disturbed. Glutathione, however, is a substance which occurs naturally in the living cell and hence results with it must have some significance in the dynamics of normal cell processes.

Warburg [1926] suggests that the behaviour of carbylamine in affecting aerobic fermentation but not respiration is due to two properties of the sub-

stance: an ability to enter into complex combinations with heavy metals and an inability to react with the heavy metal of the respiration ferment. The latter is more sensitive towards free cyanide than the catalyst of the Pasteur reaction.

Perhaps the effects of cysteine and glutathione may be interpreted in a similar manner. Cysteine is able to enter into combination both with the catalysts of respiration and of the Pasteur phenomenon, whilst glutathione can only affect the latter. This explanation, however, is not very satisfactory, for the marked inhibitory effect of cysteine on the rate of respiration in the presence of glucose is not found to occur with the respiratory rate in the presence of glycerol. If cysteine enters into combination with a respiratory catalyst it would be anticipated that all oxidations of yeast would be affected. The conclusion would be that either there is a particular catalyst which comes into play during glucose breakdown and which is affected by cysteine and not by glutathione, or that there is a particular process occurring in the aerobic breakdown of glucose by yeast whose course is affected to a much greater extent by the reducing action of cysteine than by that of glutathione. Further investigation is necessary to settle this problem.

Bumm and Appel [1932] have demonstrated that glutathione increases aerobic glycolysis by tumour cells to the limits found anaerobically, and it seems likely that this effect is closely related to the phenomenon already described.

The inference would be that glutathione, present in the living cell, plays some controlling part in the relationship between respiration and fermentation (or glycolysis) in the presence of glucose.

Preliminary experiments with brewer's yeast have shown a behaviour with glutathione similar to that experienced with baker's yeast, but the effect is not so well marked. This is to be correlated with the fact that the respiration of the brewer's yeast was found to be extremely small compared with the fermentation. Moreover, iodine titrations showed a larger content of —SH groups in the brewer's yeast than in the baker's yeast. The addition of an extract of brewer's yeast to baker's yeast was found to increase the rate of aerobic fermentation of glucose, this being probably due to glutathione present in the extract.

The fact that cysteine or glutathione has the property of securing a rate of fermentation by yeast which approximates to that obtained under anaerobic conditions recalls the observation [Quastel and Stephenson, 1926] that thiol compounds enable a strict anaerobe such as *B. sporogenes* to proliferate under aerobic conditions. The suggestion made then was that the presence of thiol compounds established a limiting reduction potential which was necessary for proliferation of this organism. In an analogous manner it may be suggested that the Pasteur phenomenon—the relationship between aerobic fermentation and respiration—is associated in the cell with the existence of a particular reduction potential, whose magnitude will depend in part on the presence of

thiol compounds. Whether this be the case or not there seems little question that thiol compounds normally play some part in determining the relationship between fermentation and respiration of yeast in the presence of glucose.

Further work on this question is now in progress.

Summary.

1. Iodoacetic acid reacts with sodium thiosulphate, cysteine and glutathione. These substances diminish the toxicity of iodoacetic acid towards biological processes.

2. Cysteine and glutathione markedly affect the relationship between fermentation and respiration of baker's yeast in the presence of glucose.

3. Cysteine inhibits the oxygen uptake of baker's yeast in the presence of glucose; glutathione has but little effect. Both thiol compounds increase the rate of CO_2 formation, glutathione having the greater action.

4. The fermentation of fructose is affected in a similar manner to that of glucose by cysteine.

5. The O_2 uptake and CO_2 output of yeast in presence of glycerol are only slightly affected by cysteine.

6. The controlling action of glutathione on aerobic fermentation is probably of significance in the normal metabolism of the cell.

We wish to express our thanks to the Medical Research Council for a grant towards the equipment of this laboratory, and for a whole time assistance grant to one of us.

REFERENCES.

Bersin (1932). *Biochem. Z.* **248**, 3.
Bumm and Appel (1932). *Z. physiol. Chem.* **210**, 79.
Krebs (1931). *Biochem. Z.* **234**, 278.
Lundsgaard (1932). *Biochem. Z.* **250**, 61.
Quastel and Stephenson (1926). *Biochem. J.* **20**, 1125.
—— and Wheatley (1932). *Biochem. J.* **26**, 725.
Warburg (1926). *Biochem. Z.* **172**, 432.

CCLVII. THE ANTISCORBUTIC POTENCY OF APPLES. V.

By MORLEY BENJAMIN CRANE
From the John Innes Horticultural Institution, Merton

and SYLVESTER SOLOMON ZILVA[1]
From the Division of Nutrition, Lister Institute, London.

(*Received November 7th, 1932.*)

Cultivated varieties of apples fall into two groups as regards the chromosome number of their nuclei, the so-called diploids with 34 and triploids with 51 chromosomes respectively. Preliminary experiments [Crane and Zilva, 1931] have shown that triploid apples reveal a tendency to possess a higher vitamin C content than diploids. The number of varieties tested in the experiments mentioned was, however, small and it was felt desirable to extend it. In this communication the results of tests carried out with a further number of triploid varieties are given and are compared with those previously obtained with triploid and diploid apples.

Experimental.

The following are the cultural details of the varieties tested.

Gennet Moyle and Ribston (East Malling).

We wish to thank Mr R. G. Hatton and Mr A. C. Painter of the East Malling Research Station, East Malling, Kent, for kindly offering this material and for supplying the cultural details connected with it.

(*a*) Age of trees—9 years.

(*b*) Stock—No. 5 (Doucin Amélioré).

(*c*) Soil—light loam over Kent ragstone—known as Hythe beds (lower greensand series).

(*d*) Manurial treatment—early years alternate dressings of London stable manure and shoddy. Later years sulphate of potash 3 cwt. per acre *plus* 6 cwt. meat and bone meal. In 1931 received 3 cwt. of sulphate of potash per acre only.

Reinette du Canada.

The bulk of this material was obtained from the Royal Horticultural Society's Gardens, Wisley, Ripley, Surrey, and we wish to thank Mr R. L. Harrow and Mr F. J. Chittenden for their help in supplying it and for the following cultural details in connection with it.

(*a*) Age of trees—approximately 25 years.

(*b*) Stock—crab stock.

(*c*) Soil—light loam over gravel.

(*d*) Manurial treatment—dressing of superphosphate of lime in the spring at the rate of approximately ¼ lb. to the square yard. Garden refuse and a dressing of sulphate of potash (2 oz. to the square yard) were forked in in 1930.

(*e*) Crop—"moderate."

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

As the quantity of material obtained from this source was not quite sufficient to complete the experiment, a further small supply was obtained from a nursery at Slough, Bucks. The cultural details for this material are as follows:

(*a*) Age of trees—5 years.
(*b*) Stock—English broadleaf.
(*c*) Soil—heavy loam.
(*d*) Manurial treatment—liberal stable manure.
(*e*) Nature of crop—generally medium.

Warner's King.

The material was obtained from a fruit farm in Kent. The cultural details are as follows:

(*a*) Age of trees—very old trees severely pruned.
(*b*) Stock—probably seedling crabs.
(*c*) Soil—medium light soil on ragstone.
(*d*) Manurial treatment—sheep-cropped but inclined to be in starved condition. Annual application of about 4 cwt. of potash salts.

Ribston (Canadian); Baldwin 1931 *and Gravenstein* 1931.

We have to thank Dr W. S. Blair of the Dominion Experimental Farm, Kentville, Nova Scotia, Canada, for this material and for the following cultural details:

(*a*) Age of trees—planted in 1912.

(*b*) Stock—French stock imported from France. Propagated in Nova Scotia at a local nursery in 1909.

(*c*) Manurial treatment—the trees since coming into bearing have been supplied with approximately a 5–9–7 fertiliser. This was used at the rate of 5 lbs. per tree in 1924, 1925 and 1926 and 7 lbs. per tree in 1927 and 1928. The fertiliser after 1928 was increased in nitrogen and a fertiliser made up of 200 lbs. of nitrate of soda, 200 lbs. of sulphate of ammonia, 300 lbs. of superphosphate and 100 lbs. of muriate of potash was applied at the rate of 8 lbs. per tree and this mixture was used at the same rate in 1930 and 1931. No farmyard manure was used. This was applied broadcast about the 10th of May, extending about 3 ft. beyond the area of the branches and on the grass as well as on the cultivated areas.

(*d*) Soil—sandy loam. These trees were clean cultivated until 10 years of age and then a strip of grass was left under the tree, varying from 2 to 8 ft. wide with trees in centre of strip, until the present. This strip of grass was clipped 2 to 3 times during the season, grass was not more than 6 ins. in height at any time. The cut material was evenly spread as a mulch under the trees. The area between this and the next tree was cultivated. The ground was fall ploughed. This cultivation was continued to July 1st at intervals of 2 weeks from the 8th May.

The rows are 40 ft. apart, every other tree is a Wagner. The above varieties are 40 ft. each way with a Wagner filler between, making all trees 20 by 40 ft.

Baldwin 1930.

One barrel ("grade 1") of this material was obtained from the United Fruit Co., Nova Scotia. No cultural details were obtained.

Gravenstein 1930.

One barrel ("grade 1") of these apples was obtained from the United Fruit Co., Nova Scotia, at the same time as the Baldwin 1930 material. In this case also no cultural details were available.

The vitamin C tests were carried out by the procedure already described [Bracewell, Hoyle and Zilva, 1930], except that owing to shortage of experimental material animals dying from intercurrent diseases could not be replaced. We are, however, of the opinion that the assessment of the degree of protection from scurvy in these cases did not suffer much in accuracy. Tables I, II and III set out the origin, the chromosome number and antiscorbutic potency respectively of the above varieties as well as of the varieties previously tested.

Table I.

Variety	Parentage	Origin
Bramley's Seedling	Not known	Raised by Mr Bramley of Southwell, Notts. Introduced in 1876
Belle de Boskoop	,,	Originated at Boskoop in Holland in 1856
Gennet Moyle	,,	Not known
Reinette du Canada	,,	Uncertain. Known in France in the eighteenth century
Blenheim Orange	,,	Raised at Woodstock near Blenheim by Mr Kempster. Introduced about 1818
Warner's King	,,	Found at Weavering in Kent at the end of the eighteenth century
Ribston Pippin	,,	Raised at Ribston Hall, Knaresborough, about 1709, from seeds brought from Rouen
Baldwin	,,	Originated about 1740 as a chance seedling on the farm of Mr Ball, Wilmington, Massachusetts
Gravenstein	,,	Said to have been found at the castle of Grafenstein, in Schleswig-Holstein. Introduced about 1760
Lane's Prince Albert	,,	Found in a garden at Berkhampstead and introduced by Messrs Lane of that town in 1857
Newton Wonder	,,	Raised by a Mr Taylor of Kings Newton, Derby, and introduced by Messrs Pearson and Co. about 1887
Cox's Orange Pippin	Alleged to be a seedling from Ribston Pippin	Raised about 1830 by a Mr Cox of Colnbrook Lawn, near Slough, and introduced by Mr Chas. Turner
King Edward VII	Said to be Blenheim Orange × Golden Noble	Introduced by Messrs Rowe of Worcester about 1908
Worcester Pearmain	Alleged to be a seedling from Devonshire Quarrenden	Originated at Swan Pool, near Worcester, by a Mr Hale. Introduced by Messrs Smith of Worcester in 1874

Table II.

	Chromosome numbers ($2n$)				
Variety	Kobel	Crane and Lawrence	Nebel	Darlington and Moffett	Rybin
Bramley's Seedling	—	51	—	51	—
Belle de Boskoop	*ca.* 46	—	51	—	—
Gennet Moyle	—	51	—	51	—
Reinette du Canada	38–40	—	51	—	51
Blenheim Orange	—	51	51	51	—
Warner's King	42	—	—	—	—
Ribston Pippin	42	—	51	51	—
Baldwin	48–49	—	51	51	—
Gravenstein	45–46	—	51	—	—
Lane's Prince Albert	—	34	—	34	—
Newton Wonder	—	34	—	34	—
Cox's Orange Pippin	34	34	—	34	—
King Edward VII	Not cytologically examined but resembles a diploid in characters				
Worcester Pearmain	—	34	—	34	—

Table III.

Daily dose of apples g.		3	5	10	20
Bramley's Seedling.	Triploid	+ + + ⊥	+ + + +	+ + + +	+ + + +
Belle de Boskoop	,,	+ + + ⊥	+ + + ⊥ to + + + +	+ + + +	+ + + +
Gennet Moyle	,,	+ + +	+ + + ⊥	+ + + +	Not tested
Reinette du Canada	,,	+ ⊥	+ +	+ + + ⊥	Not tested
Blenheim Orange	,,	⊥	+ + +	+ + + ⊥	+ + + +
Warner's King	,,	⊥	+ + ⊥	+ + + ⊥	+ + + +
Ribston, East Malling	,,	+	+ ⊥	+ + + ⊥	Not tested
Ribston, Canadian	,,	0 to ⊥	⊥	+	+ + + ⊥
Baldwin 1930	,,	⊥	+	+ + ⊥	+ + + +
Baldwin 1931	,,	0	⊥	+ + +	+ + + +
Gravenstein 1930	,,	0	0	0	⊥
Gravenstein 1931	,,	0	0	0	⊥ to +
Lane's Prince Albert.	Diploid	⊥	+ + +	+ + + +	+ + + +
Newton Wonder	,,	⊥	+ ⊥	+ + + +	Not tested
Cox's Orange Pippin	,,	⊥	+	+ + ⊥	+ + + ⊥
King Edward VII	,,	0	⊥	+ + ⊥	+ + + ⊥
Worcester Pearmain	,,	0	0	⊥	+ + +

Conclusions.

As in the case of the diploid, the triploid apples show a variation in antiscorbutic activity amongst the varieties tested. Of these at least three, Bramley's Seedling, Belle de Boskoop and Gennet Moyle, stand out as exceptionally active, and four varieties, namely Reinette du Canada, Blenheim Orange, Warner's King and Ribston Pippin are roughly of the same order of potency as Lane's Prince Albert, an outstandingly active variety of the diploid group of apples. The Gravenstein variety, although a triploid, is the least active apple registered in this investigation. It is clear that the results, although suggesting that some connection may exist between the chromosome number and the antiscorbutic activity of the apple, do not supply incontrovertible evidence to that end.

It must however be stressed in this connection that nothing of real value is known of the parentage of the apples used in this investigation, nor is there available any definite knowledge concerning the origin of the triploid form of apple in general. Triploids commonly originate from the crossing of tetraploids with diploids, but since a tetraploid form of the cultivated apple is unknown one is forced to consider the probability that triploid varieties of apples arose from diploids by the accidental functioning of an unreduced gamete. Rybin [1927], moreover, has actually reported triploids arising from diploid parents and, further, triploid forms have arisen in a similar way in other fruits [Osawa, 1920].

It is probable that a triploid derived from "selfing" a diploid with a high vitamin C content or from crossing two very active diploids would yield a triploid variety richer in vitamin C than either diploid parent and that triploids derived from low content diploids would be correspondingly less active. A similar variation in the distribution of other characters among apples is known. Vigour of growth may be cited as an example. Although triploids are

in general more vigorous than diploids this property varies amongst the individual triploid and diploid varieties.

Conclusive evidence could only be obtained by comparing the vitamin C content of triploid forms with that of the diploid apples from which they were derived. This would entail the production of the corresponding triploid forms artificially, a task which is fraught with almost insuperable difficulties. The path for further experiments obviously lies in the direction of other fruits or vegetables in which polyploidy occurs, or can be induced with greater ease, and in which strictly comparable diploid and polyploid forms can be obtained.

We are indebted to Mrs K. M. Balfour for helping us with the tests in this investigation.

REFERENCES.

Bracewell, Hoyle and Zilva (1930). *Medical Research Council Special Report Series*, No. 146.
Crane and Zilva (1931). *J. Pomology Hort. Sci.* **9**, 228.
Osawa (1920). *Bull. Imp. Ser. Exp. Sta.* Japan, **1**, 317.
Rybin (1927). *Bull. Appl. Bot. Plant Breeding*, Leningrad, **17** (3), 101.

CCLVIII. THE ANTISCORBUTIC ACTIVITY OF THE CORTEX OF THE SUPRARENAL GLAND OF THE OX.

By SYLVESTER SOLOMON ZILVA[1].

From the Division of Nutrition, Lister Institute, London.

(*Received November 10th, 1932.*)

In connection with an inquiry which has been going on for some months on the question of the identity of hexuronic acid with the antiscorbutic factor, I took the opportunity of testing the cortex of the suprarenal gland of the ox. This as will be seen from Fig. 1 was more active than decitrated lemon

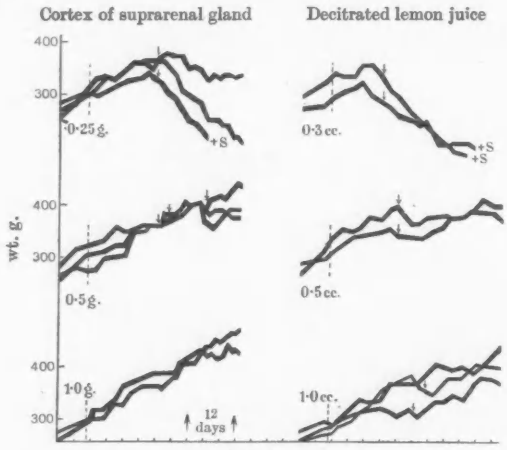


Fig. 1.

↓ = onset of scurvy.
+S = death from scurvy.
⋮ = beginning of administration of dose.

juice tested at the same time and under the same conditions. In view of the fact that Dr L. J. Harris, who was kind enough to show me his detailed results while they were in the press [see also 1932], is independently publishing his experiments on the activity of the cortex of the suprarenal gland, I think it of interest also to record my tests the outcome of which is similar to his.

REFERENCE.

Harris (1932). *Lancet*, ii, 235.

[1] Member of the Scientific Staff of the Medical Research Council working with grants for assistance and expenses.

CCLIX. PHYSICO-CHEMICAL STUDIES OF LIPOIDS IN THEIR RELATIONS TO SALTS, DRUGS AND PROTEINS[1].

By MONA SPIEGEL-ADOLF.

From the Department of Colloid Chemistry, D. J. McCarthy Foundation, Temple University Medical School, Philadelphia, Pa.

(*Received October 18th, 1932.*)

ALTHOUGH extensive investigations [Remesow, 1930] have been made recently on the physico-chemical behaviour of some lipoid sols, explanations are still lacking of some problems interesting to the biologist. Of these problems, the specific therapeutic effect of bromides and the relation of lipoids to proteins have been chosen for a physico-chemical investigation.

In a previous paper [Spiegel-Adolf, 1932], an *in vitro* analogy with the specific effect of bromides on the substance of the central nervous system was demonstrated. While the Hofmeister series of anions gives no clue in this direction, it could be shown that bromides have a stronger depressing effect on the viscosity of lecithin sol than any other salts, except iodides containing traces of free iodine. This seemed to suggest that the specific effect of bromides is not due to the bromide ions, but to free bromine. Pauli [1929] has pointed out the possibility of such a mechanism, and recent work of Lanza [1931] has proved that the addition of iodine to KI does not change the molecular concentration and only very slightly affects the electrolytic dissociation of the latter. Owing to the unsaturated fatty acid content of lecithin a reaction with bromine or iodine is possible. As there is chemical evidence that fatty acids add KI, too, and are then enabled to bind more of other substances, even the behaviour of the compounds of lecithin and protein can be explained in this way.

In order to test this hypothesis, the following series of experiments was made. Though egg-lecithin (Merck) is, according to Remesow [1930], far from pure, it was used, since most work has been done on it, and results permitting comparison with the latter were needed. The lecithin sol was made according to Keeser [1924] by adding small portions of boiling alcoholic solution of lecithin to boiling redistilled water. Some figures concerning the conductivity and c_H of such sols are reported in Table IV. In order to check the possible influence of traces of alcohol, emulsions of lecithin in redistilled water were used in several experiments. Such emulsions [*cf.* Thomas, 1915], are more opaque than a fresh sample of a sol made after Keeser, but are nevertheless stable. The protein used consisted of serum-albumin and pseudoglobulin prepared from horse-serum by $(NH_4)_2SO_4$ fractionation, and purified by reprecipitation, dialysis and electrodialysis.

[1] Partly read before the Physiological Society of Philadelphia, March 14th, 1932.

It is known that the total refraction of a solution is equal to the sum of the refractions due to the separate solutes, but this is only true if there are no chemical reactions between them. If KBr and KI containing free iodine should act on the lecithin in the way suggested above, it is to be expected that the refractive index of a mixture of lecithin sol with such salts will differ from that of the same sol with KCl, for instance. As only very slight changes were expected, the interferometer of Zeiss was used for the following investigations [*cf.* Hirsch and Kunze, 1922]. As the lecithin sol that was to be used was far from pure, and the molecular weight of the colloidal particle unknown, no attempt was made to obtain absolute values for the refraction. The 1 % lecithin sol was diluted 20 times; the final concentration of the salts was 0·01 *M*. The interferometric values were measured in the lecithin sol, the salt solution and in a mixture of lecithin sol and salt. Correction for water was made in every case. The results are given in Table I.

Table I. *1/20 lecithin sol—74* I.U., *cell 20 mm.*

Neutral salt concentration	KCl		KBr		KI		KI+I		KCNS	
	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.
0·01 %	200	199	252	238	347	339	367	349	288	284
Difference	1		14		8		18		4	

In all cases the sum of the refractions of the separate components was lower than the observed value. The effect of bromide however was by far the greatest, only being surpassed by the effect of KI containing 0·02 g. of iodine per 10 cc. of fresh *N* KI. KCl did not significantly alter the refraction, even when the concentration of the salt was raised 10 times. The absolute values of the changes in interferometric units (I.U.) are only small, but while it is possible to get exact readings to $\pm$ 1 I.U., there is, according to Marc [1912], less danger, for colloids at least, of being mistaken in the right band when using dilute solutions; in fact the readings permitted no doubt in this direction. On the other hand, the use of more concentrated solutions would not increase the sensitivity of the method, as the increased opacity would necessitate a shorter cell. The absolute values are well within the ranges that could be expected according to other findings [Hirsch and Kossuth, 1922].

As was mentioned in the previous paper, the swelling of lecithin sol is lowered to very different degrees by chloride and bromide. It seems that a part of the lowering effect on the viscosity common to both salts is due to electrical discharge of the lecithin sol, hydration being in close connection with the electrical charge. But the greater effect of bromide may be due to colloidal changes consequent on chemical reaction. In order to decide whether the observed relations between viscosity and refraction hold for other colloids, interferometric measurements were made on protein solutions in the same way as on the lecithin sol, and it was found that the two salts had practically

the same effect on the viscosity and the refraction of both serum-albumin and pseudoglobulin.

The experiments which are described in this paper confirm those previously reported, in demonstrating the specific effect of bromides. Naturally they are not sufficient to decide whether bromides act in the same way on the brain, or if they do, that this physico-chemical behaviour is the reason for their specific effect.

Winterstein [1926], considering the results of Hansteen Cranmer confirmed by Biedermann, believes that the lipoids actually present in the cells are quite different from those obtained by extraction. On the other hand, Handovsky and Wagner [1911] observed no reaction between lecithin sol and drugs in their experiments on viscosity. In order to investigate the possibility of an additional parallelism between viscosity and refraction, further experiments were made on these lines. As far as possible, the kind and concentration of the drugs used were the same as those employed by Handovsky and Wagner. Experiments were also made with sodium-luminal, the clinical use of which is similar to that of bromides. Finally, a convulsion-producing poison, strychnine nitrate, was tried in the same way. The results are summarised in Table II.

Table II. *1/20 lecithin sol—74* I.U.

1% lecithin sol diluted %	Final concentration	Interferometric units			
		Measured	Calculated	Difference	Cell mm.
1/20	0·1 % sodium-luminal*	288	271	17	20
1/20	0·15 % chloral hydrate	271	255	16	20
1/10	Sulfonal, half saturation	102	103	−1	5
1/10	Sulfonal, 9/10 saturation	137	137	0	5
1/10	Trional, half saturation	146	146	0	5
1/10	Trional, 9/10 saturation	208	211	−3	5
1/10	0·05 % urethane	302	302	0	5
1/10	0·01 % strychnine nitrate	98	98	0	5

* NaOH of the same p_H as the sodium-luminal solution has no effect on the refraction of the lecithin sol.

The figures suggest a reaction between lecithin sol and sodium-luminal, or chloral hydrate, though they may also mean that the dispersion of the lecithin is increased. The negative results with sulfonal, trional and urethane agree with the findings of Handovsky and Wagner. The opposite effect exercised by the highest concentration of trional on lecithin sol, is explained by a decrease of the dispersion of the latter. Strychnine nitrate had no effect on the refractive power of lecithin sol.

The reaction between lecithin and proteins depends largely upon the c_H of the solution, the presence of neutral salts, and the age of the lecithin sol. The first point has been extensively investigated by Rona and Deutsch [1926], but underestimation of the important rôle of salt concentration in the relation between lecithin sol and proteins seems to be the reason for contradictory opinions in this matter.

It was noted previously [Spiegel-Adolf, 1932] that electrolyte-free solutions of serum-albumin and pseudoglobulin flocculate lecithin-sol made according to the method of Keeser [1924]. In good accord with results found by Pauli and Weiss [1928] in their investigations on colloid dyes, pseudoglobulin has a stronger flocculating effect than serum-albumin. More than 50 times as much of either protein is necessary for flocculation of 24 hours-old lecithin sol than for a week-old sol. Table III records the results of some experiments of this kind.

Table III.

× = flocculation.

Concentration of protein %		1·5	0·75	0·28	0·14	0·045	0·028	0·014	0·005	0·003
Fresh lecithin sol	Serum-albumin	× × ×	0	0	0	—	0	0	0	—
	Pseudoglobulin	× ×	× ×	×	0	—	0	0	0	—
Old lecithin sol	Serum-albumin	× × ×	× × ×	× × ×	× × ×	0	0	0	0	—
	Pseudoglobulin	× × ×	× × ×	× × ×	× × ×	—	× ×	× ×	×	0

According to Freundlich [1922], the ageing of lecithin sol consists of changes by which the latter partly loses its hydrophile properties and grows more hydrophobe. In good accord with this, the amounts of protein necessary for the flocculation of aged lecithin sol are within the range of the corresponding values for gold sol [Spiegel-Adolf, 1927].

There seem to exist no systematic investigations of the physico-chemical changes during ageing of lecithin sol, though there are various hints in this direction [Porges and Neubauer, 1908; Rona and Deutsch, 1926]. For the better understanding of what has been said above and what is to follow, some observations are recorded in Table IV.

Table IV.

Age of lecithin sol (Keeser)	Conductivity rec. Ohm	Viscosity 1/5 sec.	c_H
24 hours	$1{\cdot}69 \times 10^{-5}$	1405	$1{\cdot}37 \times 10^{-4}$
8 days	$1{\cdot}84 \times 10^{-5}$	1380	—
12 days	—	—	$1{\cdot}77 \times 10^{-4}$
18 days	$2{\cdot}10 \times 10^{-5}$	—	$1{\cdot}95 \times 10^{-4}$
30 days	$2{\cdot}33 \times 10^{-5}$	—	—
Lecithin sol water emulsion 24 hours	—	—	$1{\cdot}95 \times 10^{-4}$

The above experiments were performed on lecithin sols kept exclusively in bottles of pyrex glass under optimum conditions for stability. Nevertheless, the 1 % solutions, which were first translucent and of a reddish-yellow hue, became more opaque after a few days and, in the course of time, much of their colour faded. A sample kept for a year was nearly white. If infection by moulds were avoided, the sol seemed to be fairly stable. As long as no precipitation had occurred, no changes in the refractive index of the colloid solution could be detected, and measurements of the c_H using the hydrogen electrode showed only small changes. Measurements made on the same sample immediately after preparation, and a week later, indicated a slight increase of the acidity.

After 18 days, a c_H was reached corresponding to that of a fresh watery emulsion of lecithin. The secondary increase of c_H was accompanied by a similar change of the conductivity, while the viscosity showed a decrease from the initial value.

Porges and Neubauer [1908] have already shown that the flocculation of lecithin sol by proteins is inhibited by the presence of neutral salts. This is not a property peculiar to the lecithin sol, but is observed with negative sols such as colloid solutions of gold, mastic and cholesterol. In this way, it is possible to obtain stable solutions containing known amounts of lecithin, protein, and salts. In a previous communication [1932], the viscosity of such solutions under the influence of chlorides and bromides has been investigated. Handovsky and Wagner [1911] had used similar solutions in order to ascertain whether there were any reason for assuming the formation of a compound between protein and lecithin sol, but obtained negative results. Later on, Arnd and Hafner [1926] found that the refractive power of a pseudoglobulin solution was markedly lowered by the addition of lecithin sol, which they accepted as proof of formation of a lipoid-protein compound. In order to explain the discrepancy between these opinions, experiments were started, using interferometric measurements. At the same time, the influence of different salts was tested. Some results are reported in Tables V and VI.

Table V. *Lecithin sol and protein (final concentration 0·1 %).*

NaCl concentration *N*	Serum-albumin		Pseudoglobulin	
	Observed	Calculated	Observed	Calculated
0·025	162	160	137	146
0·05	—	—	222	220
0·1	398	396	366	365
0·2	669	668	—	—

Table VI. *Pseudoglobulin and lecithin sol (final concentration 0·1 %).*

0·1 *N* final concentration	KCl		KBr		KI		KCNS		$(NH_4)_2SO_4$	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
Sol 7 days	408	413	527	524	779	776	130*	130*	88*	86*
Sol 14 days	—	—	95*	111*	—	—	—	—	—	—
Fresh 1 day	—	—	528	525	—	—	—	—	—	—

* All measurements were made in a 5 mm. cell except those marked with asterisks, for which a 1 mm. cell was used.

The figures suggest the following interpretation. As long as the salt concentration is sufficient to avoid the faintest signs of flocculation, no changes of the refractive indices are observed. Different salts seem to behave in the same way. If the salt concentration is lowered to a point at which the solution becomes unstable, then the interferometric value of the solution is lower than the sum of the corresponding values for the separate components. The results are the same whether the instability be due to an initial low salt concentration, or whether through the ageing of the colloid, the salt concentration becomes

insufficient. These findings explain the observations of Handovsky and Wagner as well as those of Arnd and Hafner [1926], though they do not necessarily confirm the conclusions of the latter authors. The decrease in refraction must be explained primarily by a decrease of soluble material.

The first row of figures in Table V indicates a difference between the behaviours of serum-albumin and pseudoglobulin towards lecithin at the same low salt concentration. While the latter is not sufficient to avoid flocculation of the pseudoglobulin-salt-lecithin mixture, it is still able to guarantee the stability of a solution in which pseudoglobulin is replaced by serum-albumin. Although there is some similarity in this respect to the behaviour of the same salt-protein mixtures toward gold sol, it is not possible to speak, in the case of lecithin sol, of the protective power of the proteins. The same amount of neutral salt which inhibits the flocculating effect of proteins, in both cases precipitates the gold sol, but leaves the lecithin sol apparently unchanged. In earlier experiments on gold sol, evidence has been found for assuming that a compound of proteins and neutral salt would be able to protect the gold sol from the flocculating effect of further salt addition. Experiments of a similar kind were started with lecithin sol, only, instead of salt addition, pseudoglobulin was tried for testing the protective power of a serum-albumin-neutral salt compound. Only in cases in which there was a certain gap between the salt concentration able to inhibit the flocculating effect of serum-albumin and that having the same influence on pseudoglobulin, are such experiments possible. For this purpose, only lecithin sol of a certain age could be used. In recently prepared samples, the differences are too slight; in very old sols, sometimes the protective power of salts, even against albumin, vanishes. Only lecithin sols 14–21 days after the preparation according to Keeser, fresh emulsions of lecithin in distilled water, or lecithin sols derived from an older, perhaps oxidised sample of lecithin, proved to be of use for the experiments in question. The results of two series are summarised in Table VII.

These results seem to show that under certain conditions, flocculating concentrations of pseudoglobulin do not show this effect in the presence of serum-albumin and neutral salt. Though the latter at the same concentration is, in itself, not sufficient to prevent the flocculation effect of pseudoglobulin, its presence is necessary for giving the serum-albumin protective power. Non-flocculating concentration of the latter in an electrolyte-free medium is unable to inhibit the precipitation of the lecithin sol by pseudoglobulin. The negative result of interferometric investigation made in order to trace some reaction between electrolyte-free serum-albumin and pseudoglobulin was in accordance with these findings. For this reason, the above-mentioned influence of the neutral salt-serum-albumin compound on pseudoglobulin cannot be directly compared with observations made by Pauli and Singer [1932], who found that ovalbumin prevented, to some extent, the flocculation of certain colloid dyes by serum-albumin in the absence of salt.

Next to the salt concentration, the protective power depends upon the

Table VII. *Part I.*

× = flocculation. + = opacity.

Lecithin sol 19 days old	NaCl final concentration *N*	Serum-albumin %	Pseudoglobulin %	Result
1/4	0·05	0	0	0
1/4	0	0·25	0	× × ×
1/4	0	0	0·25	× × ×
1/4	0·05	0·25–0·5	0	0
1/4	0·05	0	0·25–0·5	× × ×
1/4	0·05	0·25	0·25	0
1/4	0·05	0·2	0·3	0
1/4	0·05	0·15	0·35	× ×
1/4	0·05	0·1	0·4	× × +

Table VII. *Part II.*

Lecithin sol aged by boiling	KCl final concentration *N*	Serum-albumin %	Pseudoglobulin %	Result
1/4	0·1	0	0	0
1/4	0	0·75	0	× × ×
1/4	0	0	0·75	× × ×
1/4	0·1	0	0·7–1·4	×
1/4	0·1	0·75–1·4	0	0
1/4	0·1	0·75	0·7	0
1/4	0·1	0·6	0·84	0
1/4	0·1	0·45	1·0	0
1/4	0·1	0·3	1·1	× + +
1/4	0·1	0·15	1·25	× × ×

ratio of serum-albumin to pseudoglobulin. If the latter falls below 2/3–1/2, precipitation starts. It seems therefore that the behaviour of the total protein mixture toward the lecithin sol depends on the relation of its components. The similarity of this behaviour to that of serum under various pathological conditions is somewhat striking. There, too, the so-called albumin/globulin quotient [Hofmann, 1883] seems to determine, in many respects, the properties of the serum and other biological fluids. The reactions of spinal fluid toward gold sol seem in particular, according to Schmitt [1927] to depend largely on this property. An increase of globulin has been described in nearly all luetical changes. The above-mentioned results may be of some value for a further understanding of luetical reactions using the flocculation methods.

SUMMARY.

1. Confirming prior experiments on viscosity, interferometric measurements seem to give evidence for a supposed reaction between bromine and lecithin sol, but fail to explain the effect of hypnotics.

2. The effect of lecithin addition to proteins depends on the salt content of the latter. In an electrolyte-free medium, flocculation occurs; if there is an excess of salt, no changes can be detected by help of the interferometer. Between those two extremes there are salt concentrations at which lecithin-sol additions cause a decrease of the refractive index of the mixture.

3. For lecithin sols of a given age, different concentrations of neutral salts are required to avoid flocculation by the same amount of serum-albumin or

pseudoglobulin. In mixtures containing both kinds of proteins with just sufficient salt to prevent flocculation by serum-albumin, the serum-albumin-neutral salt compound seems to inhibit to some degree the flocculating power of pseudoglobulin, the final effect depending on the albumin/globulin ratio.

REFERENCES.

Arnd and Hafner (1926). *Biochem. Z.* **167**, 440.
Freundlich (1922). Kapillarchemie. (Akad. Verlag, Leipzig.)
Handovsky and Wagner (1911). *Biochem. Z.* **31**, 32.
Hirsch and Kossuth (1922). *Fermentforsch.* **6**, 302.
—— and Kunze (1922). *Fermentforsch.* **6**, 30.
Hofmann (1883). *Arch. exp. Path. Pharm.* **16**, 133.
Keeser (1924). *Biochem. Z.* **154**, 321.
Lanza (1931). *Ann. Soc. Esp. Fis. Guim.* **29**, 626.
Marc (1912). *Chem. Ztg.* **58**, 537.
Pauli (1929). *Wien. klin. Woch.* **42**, 1556.
—— and Singer (1932). *Biochem. Z.* **244**, 76.
—— and Weiss (1928). *Biochem. Z.* **203**, 103.
Porges and Neubauer (1908). *Biochem. Z.* **7**, 152.
Remesow (1930). *Biochem. Z.* **218**, 86, 134, 147, 157, 173.
Rona and Deutsch (1926). *Biochem. Z.* **171**, 89.
Schmitt (1927). *Kolloid Z.* **41**, 261, 334.
Spiegel-Adolf (1927). *Biochem. Z.* **180**, 395.
—— (1932). *Klin. Woch.* **11**, 185.
Thomas (1915). *J. Biol. Chem.* **23**, 359.
Winterstein (1926). Die Narkose. 2nd edit. (Springer, Berlin.)

CCLX. HEXOSEMONOPHOSPHORIC ESTERS: MANNOSEMONOPHOSPHATE.

By ROBERT ROBISON.

From the Biochemical Department, The Lister Institute, London.

(*Received November 11th, 1932.*)

It has been established that the hexosemonophosphate formed during alcoholic fermentation of sugars by yeast enzymes is not a single compound but consists of a mixture of aldose and ketose esters.

One, and probably the chief, component of this product was isolated by Robison and King [1931], who concluded from their own experiments and from those of King, McLaughlin and Morgan [1931] that this pure compound most probably had the structure of glucose-6-phosphate. Its specific rotation differed from that first recorded by Levene and Raymond [1930; 1931, 1] for a synthetic ester, which they considered to be glucose-6-phosphate; but the discrepancy largely disappeared as the result of further purification of the synthetic ester [Levene and Raymond, 1931, 2].

Robison and King also isolated a ketose ester similar in most respects to the Neuberg fructosemonophosphate, but having a somewhat lower reducing power (Hagedorn and Jensen); and their further analytical results pointed definitely to the presence of still other esters in the fermentation product. The isolation of one of these esters and the further investigation of the fructosemonophosphate and the residual fractions are described in the present communication.

Isolation of mannosemonophosphate.

During the separation of glucosemonophosphate, many intermediate fractions which no longer contained significant amounts of fructose esters, as judged by the Selivanoff reaction, still gave low values for the iodimetric reducing power. The full value theoretically required for an aldosemonophosphate was obtained only after repeated recrystallisation of the brucine salt from methyl alcohol. The residual fractions obtained in these final stages of the purification were recrystallised 5 times from boiling absolute methyl alcohol and yielded clusters of short needles, very different in appearance from the soft, opaque, silky crystals of pure brucine glucosemonophosphate. These needle fractions were converted into the barium salt, which was purified and analysed by the methods described in previous papers. The results are shown in Table I (salt No. 4), the corresponding values for a typical fermentation hexosemonophosphate (No. 1) and for pure glucosemonophosphate

Table I. *Analyses of barium salts of hexosemonophosphoric acids obtained from the products of fermentation by yeast juice.*

No. of salt	Source of the barium salt	P %	Fructose (Selivanoff) %	Reducing power as glucose (%) H. and J.*	Reducing power as glucose (%) Iodine	Aldose-monophosphate %	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5461}^{20°}$ of free acid
1	Hexosemonophosphate	7·85	6	30·0	25·2	55	+14·4°	+29·5°
2	Glucosemonophosphate	7·86	0·5	35·5	45·7	100	+20·6° (c=0·84 %)	+41·4° (c=0·74 %)
3	Fructosemonophosphate (Neuberg) prepared from highly purified hexosediphosphate	7·80	22	36·2	3·0	7	+ 0·7°	—
4	Brucine salt of new ester recrystallised 5 times from absolute methyl alcohol	7·82	3	36·3	27·5	60	+ 3·1° (c=1·4 %)	— —
5	Residual salt after 17 hours' hydrolysis (28 %) of No. 4 with N HCl at 100°	7·20	1	34·0	25·6	56	+ 3·4° (c=0·4 %)	—
6	Brucine salt from No. 4, recrystallised 5 times from 95 % methyl alcohol	7·82	2	36·9	27·0	59	+ 3·6° (c=0·7 %)	+15·1° (c=1·7 %) +17·0° in 0·1 N H_2SO_4
7	Second fractions from No. 6 recrystallised 4 times from 90 % methyl alcohol and once from water	7·69	1	35·5	30·5	67	+ 3·4° (c=0·7 %)	
8	Another preparation similarly purified	7·65	1·5	36·2	28·0	61	+ 3·1° (c=0·5 %)	
9	Fructosemonophosphate isolated from fermentation hexosemonophosphate	7·73	22	34·9	1·6	3	+ 2·3° (c=0·8 %)	—
10 and 11	Brucine salts obtained in the fractionation of hexosemonophosphate	7·78 7·29	7 4	23·9 20·1	12·6 14·7	28 32	+10·8° +12·7°	—

$C_6H_{11}O_9PO_4Ba$ requires P, 7·85 %; $C_6H_{12}O_6$, 45·6 %.

* Determined with addition of 0·5 cc. of 0·5 N NaOH.

and fructosemonophosphate (Neuberg) being given in the same table for comparison. While the phosphorus content and H. and J. reducing power were the same as for glucose- and fructose-monophosphates, the new compound differed markedly from both these esters in other respects. The iodimetric value was only 60 % of that required for an aldosemonophosphate, but the low Selivanoff value precluded the presence of fructose derivatives in large amount. The low specific rotation also distinguished the new ester sharply from glucosemonophosphate.

Examination by fractional acid hydrolysis. 0·3 g. of this salt, No. 4, was heated with N H_2SO_4 at 100° for 17 hours, the hydrolysis amounting to 28 %; the residual ester was freed from inorganic phosphate and analysed (salt No. 5). The Selivanoff value was still lower (1 %), but the remaining values were not significantly changed from those of the original salt. (The quantity recovered was inadequate for thorough purification and the phosphorus content was somewhat low.)

Further recrystallisation of the brucine salt. The remainder (1·5 g.) of salt No. 4 was reconverted into brucine salt, which was again recrystallised 5 times from 95 % methyl alcohol. Second crops obtained from the mother-liquors were recrystallised 4 times from 90 % methyl alcohol and once from

water at 40°. Analyses of the barium salts prepared from these two fractions are given in Table I (Nos. 6 and 7). No. 8 was a barium salt obtained by similar methods from another hexosemonophosphate preparation.

The properties of these salts suggested that they consisted, wholly or to a very large extent, of a new ester, possibly a mannosemonophosphate.

Hydrolysis of the ester by 0·1 N H_2SO_4.

A quantity equivalent to 1 milligram mol. of the free ester was dissolved in 0·1 *N* H_2SO_4 (17 cc.) and heated in a boiling water-bath, the amount of hydrolysis being estimated from time to time. The hydrolysis was stopped after 93 hours, when 79·6 % of the total phosphate had been set free. The solution, which was then dark brown in colour, was neutralised with baryta, treated with twice its volume of alcohol to precipitate the free and combined phosphates and filtered. The filtrate containing the free sugar was evaporated rapidly on a water-bath to a small volume ($p_H < 7·0$), after which the drying was completed in an evacuated desiccator. The residue was extracted repeatedly with boiling 80 % alcohol and the filtered solution evaporated to dryness as before. The rate of hydrolysis and the properties of the sugar produced are shown in Table II.

Table II.

Time (hrs.)	Hydrolysis %	$k \times 10^3$	The syrupy product gave	
4	6·9	0·13	Total P	0·4 mg.
31	43·5	0·13	Hexose (H. and J. reduction)	85 "
93	79·6	0·12	Aldose (I reduction)	89 "
			$[\alpha]_{5461}$ calculated on hexose	+15°

The rate of hydrolysis was exactly the same as that for the hydrolysis of pure glucosemonophosphate under similar conditions ($k = 0·13 \times 10^{-3}$). Some decomposition of sugar had evidently occurred, but assuming that the decomposition products were optically inactive the specific rotation, calculated on the reducing hexose, was in fair agreement with that of mannose (+ 17·1°).

The sugar solution on treatment with pure phenylhydrazine dissolved in acetic acid gave at once, in the cold, a crystalline derivative; this was recrystallised twice from 80 % ethyl alcohol. The crystals closely resembled those of mannosephenylhydrazone, which was prepared for comparison. They melted[1] at 200–201°; the specimen of mannosephenylhydrazone also melted at 200–201°, as did an intimate mixture of the two substances. Beilstein, Supplement I, gives the M.P. of mannosephenylhydrazone as 197–201°.

Phenylhydrazine derivatives of the new ester.

Phenylhydrazone. A solution (6 cc.) containing 100 mg. of the free ester was treated at room temperature with 0·2 cc. phenylhydrazine dissolved in acetic acid. A pale yellow crystalline precipitate formed within a few minutes;

[1] These melting-points were determined in the way described by Robison and King [1931, p. 329].

but the solution was allowed to remain at 0° overnight before filtration. The product was recrystallised twice from warm 75 % ethyl alcohol, from which it separated quickly at 0° in very pale yellow crystals, M.P. 144–145°.

Analysis. After being dried over sulphuric acid the substance did not lose weight in 1½ hours at 79° in the Pregl drier.

It gave P, 6·82 %.

Calculated for $C_{18}H_{27}O_8N_4P$, P, 6·78 %.

The phosphorus content corresponded with that of the phenylhydrazine salt of the phenylhydrazone of hexosemonophosphoric acid.

Phenylosazone. A solution (6 cc.) containing 100 mg. of the free ester was treated with 0·5 cc. phenylhydrazine dissolved in acetic acid, and heated in a boiling water-bath for 1 hour. After cooling to 0° the precipitate was filtered off and the solution again heated for 1 hour, cooled and filtered. The combined precipitates were recrystallised twice from boiling ethyl alcohol to which an equal volume of boiling chloroform was added, the solution being then left at 0° for 24 hours. It separated in fine yellow needles; M.P. 154–155°.

Analysis. The substance, dried over sulphuric acid, gave P, 5·69 %.

Calculated for $C_{24}H_{31}O_7N_6P$, P, 5·68 %.

The phosphorus content corresponded with that of the phenylhydrazine salt of the osazone of hexosemonophosphoric acid.

A specimen of osazone prepared from fructosediphosphate and similarly purified also melted at 154–155°, as did an intimate mixture of the two substances. It was, therefore, probable that this derivative of the new ester was identical with the osazone obtained from fructosediphosphate, *i.e.* the phenylhydrazine salt of the osazone of fructosemonophosphate (and of glucosemonophosphate).

The properties of the sugar obtained from the ester by acid hydrolysis confirmed the supposition that the compound was a monophosphoric ester of mannose; and the formation of a sparingly soluble phosphohydrazone also accorded well with this view. The identification of osazones by their melting-points is not, perhaps, conclusive; but such evidence pointed strongly to the identity of the osazone of the mannose ester with that obtained from hexosediphosphate. This would imply that the position of the phosphate group is the same in both compounds and that the new ester is probably mannose-6-phosphate.

Comparison of the rates of hydrolysis of mannosemonophosphate and glucosemonophosphate.

It has been shown that the hydrolysis of the new ester by 0·1 *N* H_2SO_4 proceeded at the same rate as that of glucosemonophosphate under similar conditions. The rates of hydrolysis of the two esters were further compared in the following experiments.

Hydrolysis by N HCl at 100°. The ester solutions were prepared by dissolving 85 mg. of the air-dry barium salts together with 40 mg. of K_2SO_4 in 20 cc. of

N HCl and centrifuging at once, to remove barium sulphate. Quantities of about 1·3 cc. of the solutions were introduced into a number of small, thin-walled, glass tubes, previously drawn out, which were then sealed up and dropped into a bath of vigorously boiling water. The bath was kept loosely covered so that the temperature was maintained at 100°. Two tubes were removed at each of the stated intervals and the inorganic phosphate was determined colorimetrically in 1 cc. or 0·5 cc. of each of the solutions. The total P was estimated in the original solution and again at the end of the hydrolysis. The concentration of the ester in each case was approximately 0·01 *M*.

The results, set out in Table III, show that the mannose ester was hydrolysed at a slightly faster rate than glucosemonophosphate and that this rate was maintained during the entire period in which 94·5 % of the phosphate was set free. The values of k are calculated for a unimolecular reaction,

$$k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2},$$

t being the time in minutes.

Table III. *Hydrolysis in* N *HCl at 100°.*

	Glucosemonophosphoric acid (0·301 mg. P per cc.)		Mannosemonophosphoric acid (0·287 mg. P per cc.)	
Time hrs.	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	12·7	0·25	16·8	0·33
8	21·6	0·20	28·7	0·28
20	44·9	0·21	53·9	0·26
45	75·0	0·23	82·5	0·28
70	88·9	0·24	94·5	0·33

Autolysis of the free esters at 100°. Ester solutions of similar concentration (0·01 *M*) were prepared by dissolving the barium salts in water containing the exact equivalent of sulphuric acid and centrifuging. The method of heating was the same as that described above.

The results, given in Table IV, show that the hydrolysis of both esters occurred in these solutions at exactly the same rate, and, further, that this rate was nearly the same as that for the hydrolysis in *N* HCl. In the case of glucosemonophosphate the hydrolysis was actually more rapid in the ester solution containing no additional acid than in *N* HCl, which is in agreement

Table IV. *Autolysis in solutions of the free esters at 100°.*

	Glucosemono-phosphoric acid (0·285 mg. P per cc.)		Mannosemono-phosphoric acid (0·306 mg. P per cc.)		Fructosemono-phosphoric acid (0·294 mg. P per cc.)	
Time hrs.	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	14·1	0·28	14·0	0·27	18·8	0·37
24	60·6	0·28	61·2	0·29	77·8	0·46
48	85·3	0·29	85·0	0·29	96·2	0·53

with the previous findings of Robison and King [1931]. The value of k for the autolysis of glucosemonophosphate is somewhat higher than that found previously for more concentrated solutions (0·03 M) by Robison and King, who observed that the value rose as the hydrolysis proceeded.

Isolation of fructosemonophosphate from the hexosemonophosphate of fermentation.

Fractional crystallisation of the brucine salts from further preparations of the fermentation hexosemonophosphate again yielded salts approximating in their properties to the Neuberg fructosemonophosphate. As these still contained a small proportion of aldose esters the barium salts were submitted to oxidation by bromine in the presence of baryta, followed by partial precipitation from their aqueous solutions by alcohol, to remove the sparingly soluble phosphohexonates.

The analytical values obtained for the purified salt agreed closely with those for the Neuberg fructosemonophosphate, the H. and J. reducing power being now within the limits found for the pure ester. It should be noted that somewhat variable values have been recorded for the specific rotation of the Neuberg ester, which is obtained by partial hydrolysis of hexosediphosphate; and this may possibly be accounted for by the presence of traces of the second hydrolysis product, which is strongly laevorotatory [Macleod and Robison, 1932].

Hydrolysis of fructosemonophosphate by acid.

The hydrolysis of the fructosemonophosphate isolated from the fermentation product was investigated under similar conditions to those employed for the aldose ester.

Hydrolysis in N *HCl at 100°.* The ester solution, of approximately 0·01 M concentration in N HCl was prepared, and the hydrolysis carried out in the manner described, except that the time intervals were much shorter. The results are set out in Table V and show that hydrolysis proceeded at a constant rate during 3 hours while over 83 % of the phosphate was liberated. The final value (92·5 %) gives a slightly lower rate; but it should be noted that the

Table V.

Time hrs.	Fructosemonophosphate (0·287 mg. P per cc.) Hydrolysis %	$k \times 10^3$	Lohmann's values for the Neuberg ester Hydrolysis %	$k \times 10^3$
0·5	24·4	4·05	20·1	3·23
1	45·3	4·68	36·9	3·23
1·5	59·6	4·39	—	—
2	70·3	4·45	62·5	3·76
3	83·6	4·30	75·0	2·93
5	92·5	2·83	84·9	1·81
8	—	—	87·4	0·44
13	—	—	90·4	0·39
33	—	—	93·0	0·14

possible errors in the P determinations have a very marked effect on the value of k when hydrolysis is approaching completion.

In the same table are given Lohmann's [1928] values for the hydrolysis of the Neuberg ester by N HCl under conditions similar to those here described. Lohmann, who applied this method to the quantitative examination of the hexosephosphates, concluded that the Neuberg monophosphate, like the parent hexosediphosphate, consisted of a mixture of esters; and his figures certainly justify this conclusion for the preparations which he examined. It is obvious that, if the hexosediphosphate contains any esters only slowly hydrolysable by acids, these would accumulate in the monophosphate fraction, obtained from it by partial hydrolysis, which represents the Neuberg ester. The question, therefore, resolves itself into the degree of purification of the hexosediphosphate or what shall be considered to constitute this fraction. Preparations of this ester, which have been very highly purified in this laboratory, show little indication, from their hydrolysis curves, of the presence of other compounds; but some evidence has emerged that other esters, forming barium salts of similar solubility to barium hexosediphosphate, may occur in the fermentation products.

It is clear from the rates of hydrolysis that the fructosemonophosphate, whose isolation from hexosemonophosphate is here described, contained a much higher proportion of the main constituent than Lohmann's ester. This is shown both by the higher initial value found for k and by the persistence of this value until a larger proportion of the total ester had been hydrolysed. It may be inferred that the substance contained less than 5 % of other esters[1].

Autolysis of the free ester at 100°. The rate of hydrolysis of the free fructosemonophosphoric acid in approximately 0·01 M solution containing no additional acid was also determined. The results, which are given in Table IV, show a quite different behaviour of this ester from that of the two aldosemonophosphates. The rate of hydrolysis in absence of HCl is, initially, less than one-tenth of the rate in N HCl, but increases somewhat as the hydrolysis proceeds. One must conclude that, in the case of the aldose esters, two essentially different compounds are being hydrolysed in the solutions of different acidity, while this is not so in the case of the fructose ester. The similarity in the values of k for the three esters, in 0·01 M solutions containing no additional acid, suggests that the compounds present in these solutions are of similar type, while, in presence of N HCl, the aldose esters pass over into another type, which is hydrolysed much less rapidly. The fructose ester cannot, or does not so readily, change into the second type and its rate of hydrolysis consequently increases with increasing hydrogen ion concentration.

Since the phosphate group is probably in the same position, 6, in the three esters, the formation of the stable 1:5 oxygen bridge is possible in glucose-

[1] Subsequent fractional precipitation of this product from aqueous solution by alcohol removed further traces of a sparingly soluble salt, probably phosphohexonate. The soluble portion was now hydrolysed by N HCl to the extent of 96·3 % in 5 hours and 98·3 % in 7 hours.

and mannose-monophosphates, but not in fructosemonophosphate. It is conceivable, therefore, that the easily hydrolysable forms present in the aqueous solutions of all three esters may represent either the furanose derivatives or possibly the free aldehydes or ketone, while the less readily hydrolysable compounds formed in N HCl or H_2SO_4 may be pyranose derivatives containing the stable oxygen ring.

It may be recalled that the phospho-osazone common to all three esters which contains no oxygen bridge is hydrolysed by N H_2SO_4 even more rapidly than fructosemonophosphate [Robison and King, 1931]. The investigation of the hydrolysis of these esters and their derivatives is being continued and may throw more light on their structure and on the causes of their reactivity.

Other facts which may be related to the existence of these dual forms are (*a*) the marked increase in the specific rotation of the glucose and mannose esters, especially the latter, on liberation from their salts, and the further increase shown in presence of 0·1 N and N H_2SO_4, (*b*) the very low iodimetric reducing power of the mannose ester. This has not yet been satisfactorily accounted for. Slightly higher values than those shown in Table I were obtained by varying the conditions of the oxidation, particularly by increasing the concentration of potassium iodide in the iodine solution; but the highest figure corresponded with only 70 % of that theoretically demanded.

Other constituents of the fermentation hexosemonophosphate.

Some progress has been made towards the isolation of the fourth ester, which is responsible for the low H. and J. reducing power of the fermentation hexosemonophosphate, compared with that of the three known components.

This ester becomes concentrated in the final mother-liquors during the crystallisation of the brucine salts. These mother-liquors were evaporated to dryness, the residues recrystallised from methyl alcohol, the mother-liquors again evaporated and these operations repeated. A crystalline fraction, obtained by adding ether and light petroleum to some of these alcoholic mother-liquors, gave the barium salt No. 10, whose analysis is shown in Table I, while No. 11 was obtained from mother-liquors at a similar stage.

Attempts to purify the brucine salt of this ester by repeated crystallisation from various solvents gave products of higher reducing power. These salts are not yet homogeneous but certainly contain a high proportion of a new ester whose reducing power is very much lower than that of the three known hexosemonophosphates.

Fractional precipitation of the barium salt by alcohol or of the lead and basic lead salts gave products differing in little except the $[\alpha]_{5461}$, which was higher in the more soluble fractions. This may have been due to traces of trehalosephosphate.

Investigation by fractional acid hydrolysis, by bromine oxidation and other methods is being continued. One property common to these fractions may be mentioned. When heated with orcinol and hydrochloric acid a green colour is

developed, but the absorption band of the amyl alcohol solution is not identical with that given by pentoses, but is situated further towards the red end of the spectrum. The salts contain only traces of nitrogen as determined by a micro-Kjeldahl method.

Discussion.

The isolation of a mannosemonophosphate from the products of alcoholic fermentation fulfils an expectation long held in these laboratories. It was shown by Harden and Young [1909] that the fermentation of mannose as well as that of glucose and fructose by yeast juice responded to the addition of phosphate and that the same hexosediphosphate was formed from all these sugars [Young, 1909]. The deduction that at some stage in the esterification the three hexoses pass through a form which is common to them all leads to the further conclusion that, since monophosphoric esters of glucose and fructose are simultaneously formed from both these sugars, a corresponding ester of mannose should also occur among the products of their fermentation.

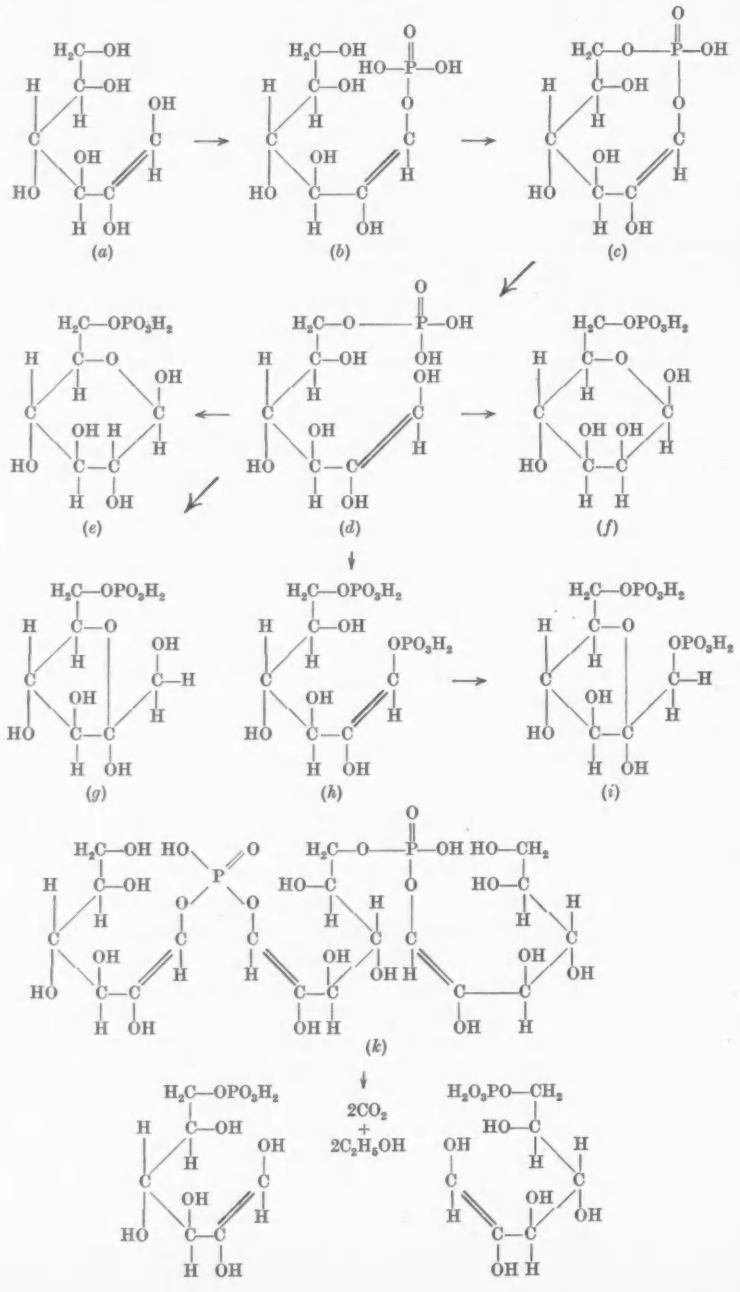
A scheme which has been suggested by the author [Robison, 1932] to account for the formation of these esters may be briefly recapitulated here. It is based on Armstrong's [1904] suggestion that the substance actually fermented by yeast is the enolic compound common to glucose, fructose and mannose. It is supposed that this enolic compound (*a*) (p. 2200) reacts rapidly with phosphoric acid, one molecule of which becomes attached to carbon atom 1 (*b*). This phosphoric group then wanders to carbon atom 6 *via* the intermediate compound (*c*) (*cf.* the wandering of the acetyl group in 1:2:3:4-tetra-acetyl-β-glucose under the influence of alkali [Fischer, 1920; Haworth, Hirst and Teece, 1930]). The resulting ester (*d*) by the addition of water to carbon atoms 1 and 2 would yield hydrated forms of glucose-, mannose-, and fructose-phosphates. Subsequent removal of water from the hydrated glucose and mannose esters could give 6-phosphoglucopyranose (*e*) and 6-phosphomannopyranose (*f*) while the hydrated fructose ester could yield only the γ-derivative, 6-phospho-fructofuranose (*g*).

The enolic group of ester (*d*) could, however, react with a further molecule of phosphoric acid, giving the diphosphoric ester (*h*) which would yield 1:6-diphosphofructofuranose (*i*), the hexosediphosphate of Harden and Young.

During the hydrolysis of these esters by phosphatases (of yeast or of bone) this series of changes may be supposed to take place in the reverse direction.

This scheme although it explains the formation of the phosphoric esters of fermentation does not account for the decomposition of half a molecule of hexose which accompanies the introduction of each phosphoric group during the fermentation.

It is conceivable that three molecules of hexose become linked together through two molecules of phosphoric acid and that the complex ester (*k*) so formed then breaks down, yielding two molecules each of hexosemonophosphate, carbon dioxide, and alcohol.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(k)
2CO2
+
2C2H5OH

Two molecules of monophosphate could similarly become linked with another molecule of hexose through two additional molecules of phosphoric acid, and this new complex ester, on disruption, would yield two molecules each of hexosediphosphate, carbon dioxide, and alcohol. The ratio

$$\frac{CO_2 \text{ produced}}{PO_4 \text{ esterified}}$$

would then be equal to unity for both stages of esterification.

We have, however, at present no evidence to prove that these assumptions are correct. The isolation and identification of the still unknown esters in the fermentation products may help us to answer this question.

The mannosemonophosphate described in this paper was obtained from the mixed products of fermentations of glucose and of fructose, so that further evidence is required to prove that it is formed from each of these sugars. The investigation of the ester was strictly limited by the small amount available and more work and more analyses are necessary to substantiate the conclusions arrived at. It is probable that the purest products were almost free from fructosemonophosphate. The absence of small amounts of glucosemonophosphate is more difficult to prove[1].

The purification of the ester by means of its phenylhydrazone is being attempted and should yield a homogeneous product. Fortunately, a much easier method has been found for the preparation of the ester than the very laborious fractionation hitherto carried out. Experiments by Dr Jephcott and the author on the fermentation of mannose by dried yeast in presence of phosphate have shown that at high temperatures (37°) esterification proceeds in a somewhat abnormal manner; a very large proportion of the total phosphate is converted into hexosemonophosphate which consists almost entirely of the mannose ester.

An account of these experiments will shortly be published and the investigation of the relationship between this abnormal esterification and production of CO_2 is being continued. It is apparent that such abnormal esterification would require some modification in the scheme outlined above.

The fermentation and esterification of mannose at 37° by fresh yeast in presence of toluene has been investigated by I. Neuberg and Ostendorf [1930], who also observed a greatly increased production of the monophosphate fraction compared with the amount formed from glucose. The products isolated by these authors, however, showed a high dextrorotation, $[\alpha]_D$, + 45° and + 34°, for the free acid, and a low P content (7·19 %). It may be suggested that this compound possibly contained mannosemonophosphate mixed with trehalosemonophosphate. The particular sample of dried yeast used by Jephcott and Robison produced much less trehalose from glucose and fructose than did those previously examined and this may have accounted, to some extent, for the purity of the mannose ester which they obtained.

[1] The synthesis of mannose-6-phosphate is at present being attempted by Mr V. N. Patwardhan and the author.

SUMMARY.

1. A third component has been isolated from the hexosemonophosphate produced by fermentation of hexoses with yeast juice. This compound has been shown to be a monophosphoric ester of mannose.

2. The $[\alpha]^{20°}_{5461}$ of the free ester is + 15·1°, and of the barium salt + 3·5°. The H. and J. reducing power of the ester is the same as that of glucose- and fructose-monophosphate. The iodimetric reducing power is only 60–70 % of the value theoretically required for an aldosemonophosphate.

3. The ester yields a sparingly soluble phenylhydrazine salt of a phenylhydrazone (M.P. 144–145°), and a phenylhydrazine salt of an osazone (M.P. 154–155°), which appears to be identical with that obtained from fructose-diphosphate and from glucose- and fructose-monophosphate.

4. From this it is inferred that the phosphate group is in the same position in all these esters, probably position 6.

5. The ester is very slowly hydrolysed by *N* HCl at 100° ($k = 0{\cdot}29 \times 10^{-3}$); the rate is slightly higher than that for glucosemonophosphate in *N* HCl at 100°, and is practically the same as the rate of hydrolysis of both the free esters in 0·01 *M* solution, containing no additional acid. The sugar produced by acid hydrolysis has the character of *d*-mannose.

6. The presence of the Neuberg fructosemonophosphate as a component of the fermentation hexosemonophosphate has been confirmed; and this ester has been isolated in purer form than that previously reported by Robison and King.

7. The hydrolysis of this fructosemonophosphate by acids at 100° has been examined. In *N* HCl the value of k was found to be $4{\cdot}36 \times 10^{-3}$, but in 0·01 *M* solution of the free ester the initial value of k was $0{\cdot}37 \times 10^{-3}$, only slightly higher than that of glucose- and mannose-monophosphate.

8. The bearing of these rates of hydrolysis on the structure of the three esters is discussed.

9. The reactions leading to the formation of fructosediphosphate and of the three monophosphates during the fermentation of hexoses are discussed, and a scheme is suggested to explain the facts at present known.

REFERENCES.

Armstrong, E. F. (1904). *Proc. Roy. Soc. Lond.* **73**, 516.
Fischer (1920). *Ber. deutsch. chem. Ges.* **53**, 1621.
Harden and Young (1909). *Proc. Roy. Soc. Lond.* B **81**, 336.
Haworth, Hirst and Teece (1930). *J. Chem. Soc.* 1405.
King, McLaughlin and Morgan (1931). *Biochem. J.* **25**, 310.
Levene and Raymond (1930). *J. Biol. Chem.* **89**, 479.
—— —— (1931, 1). *J. Biol. Chem.* **91**, 751.
—— —— (1931, 2). *J. Biol. Chem.* **92**, 757.
Lohmann (1928). *Biochem. Z.* **194**, 306.
Macleod and Robison (1932). *Chem. Ind.* **51**, 913.
Neuberg, I. and Ostendorf (1930). *Biochem. Z.* **221**, 154.
Robison (1932). Herter lectures. (New York University Press.)
—— and King (1931). *Biochem. J.* **25**, 323.
Young (1909). *Proc. Roy. Soc. Lond.* B **81**, 528.

CCLXI. THE USE OF PURE PHOSPHOTUNGSTIC ACIDS IN THE PRECIPITATION OF BASES.

I. METHOD OF CHECKING THE PURITY OF 1:24-PHOSPHOTUNGSTIC ACID.

II. THE INFLUENCE OF HYDRION CONCENTRATION AND SOME OTHER FACTORS ON THE PRECIPITATION OF BASES.

By HAROLD BARNES and RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(*Received November 2nd, 1932.*)

Introduction.

With the exception of fractionation of phosphotungstates of bases by acetone-water mixture, no attempts seem to have been made to modify the use of phosphotungstic acid [Drechsel, 1887]. It has been shown by one of us [Peters, 1930] that some bases are characterised by the hydrion concentration at which precipitates appear with neutralised phosphotungstic acid (sodium phosphotungstate). Extended use was made of these observations in concentrating vitamin B_1 by Kinnersley and Peters [1930]. As the methods there employed seemed likely to have general application, a further study was undertaken.

The primary object of our work was practical, but it was hoped also to clear the ground for theory as to the conditions governing precipitation and composition of the precipitates. Much has been learnt, but it is clear that the work only constitutes a preliminary survey of a complicated field. Fundamental observations upon the separation of complexes at various c_H are being made by Britton and his colleagues [1932], to which this problem is clearly related. Previously, the purest commercial acid had been used. Here we have concentrated mainly upon the 1 : 24-phosphotungstic acid, though some observations have been made with the 1 : 18-acid. The results are not the same as those with the commercial acids.

PART I. METHOD OF CHECKING THE PURITY OF 1:24-PHOSPHOTUNGSTIC ACID (By H. BARNES).

Phosphotungstic acid, even of the best commercial grades, is a mixture, usually considered to approximate to 10 % of the 1 : 18-acid and 90 % of the 1 : 24-acid. Throughout, the ratios expressed refer to the ratio $P_2O_5 : WO_3$.

Among the numerous acids described in the literature [Scheibler, 1872; Sprenger, 1880; Kehrmann, 1887; Kehrmann and Fraenkel, 1891; Brandhorst and Kraut, 1888; Gibbs, 1877; Péchard, 1889], 1 : 12, 1 : 16, 1 : 21, 1 : 22, 1 : 18 and 1 : 24, the last two are the most clearly established. They have been prepared for the purpose of this work by the method of Wu [1920] from Kahlbaum's highest grade of sodium tungstate. A less reliable sample upon one occasion gave a 1 : 24-acid of green-yellow tint, possibly owing to the presence of sodium molybdate [see Folin and Marenzi, 1929]. The final product was best recrystallised from a small amount of water allowed to evaporate over sulphuric acid in a desiccator at room temperature, giving large octahedra. The usual yield of the purest acid from 200 g. of tungstate was 20 g. of the 1 : 24-acid. The yield was improved upon one occasion by cooling all the reagents thoroughly at the ether extraction stage. It was found important to store the crystals in a closely stoppered bottle to prevent the efflorescence which easily alters their composition. Even with considerable precautions the original analysis figures were not given after storing for a few weeks.

Only one form of the 1 : 18-acid was used in this work, obtained from an ammonium salt like Wu's B form by ether extraction, and consisting of pale greenish-yellow thick rhombohedral plates.

Analysis of the samples used.

In order to check the purity of the 1 : 24-acid used, a new method was devised, after unsuccessful work with other analytical methods. A short description of the experiments best indicates the difficulties and may be helpful to others. No success was obtained by the application of colorimetric phosphate methods to mixtures of sodium tungstate and potassium phosphate.

The following stages can be recognised in most previous work.

(*a*) The decomposition of the complex acid. Different investigators have used widely varying reagents from 20 % boiling sodium hydroxide for 30 minutes to dilute hydrochloric acid. Evidence is scanty for the efficacy of any particular method. Experiments performed during the progress of this research suggest that different acids require different treatment for disintegration.

(*b*) Precipitation (i) of the two acids together as a mixture of mercurous tungstate and mercurous phosphate; (ii) of the phosphate as magnesium pyrophosphate; (iii) of the tungstate as tungstic oxide, or as a compound with (iv) tannic acid or (v) benzidine.

Gibbs's method [1880][1]. After decomposition the tungstic and phosphoric acids were precipitated together in boiling acid solution with mercurous nitrate, as a mixture of mercurous tungstate and mercurous phosphate, the excess acid was neutralised with mercuric oxide, and the needles so obtained after washing with dilute mercurous nitrate solution and igniting gave the sum of P_2O_5 and WO_3. Gibbs states that no phosphorus was lost during the ignition, but von Knorre [1908] considers that some phosphorus was left in the filtrate from the mercury precipitation. On a second portion Gibbs determined the phosphate as $Mg_2P_2O_7$, after precipitation as magnesium ammonium phosphate. This latter method was worked out for Gibbs by Gooch [1879] and it is clear from his results that the separation of phosphoric and tungstic acids in this way is a matter of considerable difficulty. The phosphate always seems to be in error by + 0 to 8 % due to the adsorption of sodium tungstate from the solution on to the precipitate. This error is only slightly reduced, by taking up the magnesium ammonium phosphate precipitate in hydrochloric acid, and reprecipitating with magnesia mixture. Gibbs determined the WO_3 by difference.

[1] The earlier worker [Scheibler, 1872], has not given a method of analysis.

The rather unsatisfactory nature of these methods was shown by a re-investigation of the effect of sodium tungstate on the estimation of potassium hydrogen phosphate (Sørensen), as $Mg_2P_2O_7$, after precipitation as magnesium ammonium phosphate. The original work of Gooch was amply confirmed—the error in the first precipitation always being of the order of 10 to 20 %. Several reprecipitations were then tried, and only after four such reprecipitations of the magnesium ammonium phosphate could the error be reduced to approximately 1 %. In the experimental procedure adopted, the mixtures of sodium tungstate and potassium hydrogen phosphate were made up in amounts approximately equivalent to a 1 : 20-phosphotungstic acid, the solution was heated to boiling and magnesia mixture cautiously added with constant stirring. After cooling an excess of ammonia was added, and the mixture allowed to stand at least 4 hours before filtration. Table I gives the results.

Table I.

Amount of $Na_2WO_4, 2H_2O$ taken g.	Amount of KH_2PO_4 taken g.	No. of precipitations	$Mg_2P_2O_7$ found g.	KH_2PO_4 corresponding to $Mg_2P_2O_7$ found g.	Errors %
1·5641	0·0902	1	0·0876	0·1071	18·7
1·7697	0·1099	4	0·0912	0·1114	1·4
1·0718	0·1099	4	0·0914	0·1117	1·6

Naturally such a method is very tedious and subject to considerable manipulative error.

Other methods. Kehrmann [1887], after boiling the complex acid for half an hour with sodium hydroxide, precipitated the phosphate as magnesium ammonium phosphate, using magnesium and ammonium nitrates instead of the corresponding chlorides and, after evaporating down the ammoniacal filtrates, precipitated the tungstic oxide from them as such by addition of hydrochloric acid. Sprenger [1880] proceeded in a similar manner, but used tannic acid to precipitate the tungstic oxide.

A critical survey of the literature with some experimental work was published by von Knorre [1908]. He considered the method of Kehrmann for splitting up the complex acid, namely boiling with sodium hydroxide for half an hour, as far too vigorous, and he himself appears merely to have added hydrochloric acid. He also confirmed Gooch's experience on the difficulty of obtaining a pure magnesium ammonium phosphate. He states without giving experimental details that the determination of tungsten as oxide is not accurate in the presence of magnesium salts. With regard to the tannic acid method of Sprenger, von Knorre's experiments show that, using pure solutions of sodium tungstate, the results are always too low. In addition the exact conditions for a good precipitation by this method are difficult to obtain—the appearance of colloidal solutions being frequent. Von Knorre himself precipitates the tungstate first as benzidine tungstate in acid solution, adding a little sulphuric acid to help coagulation of the precipitate. On ignition the

benzidine tungstate gives tungstic oxide, and benzidine sulphate is volatilised. Phosphate is determined in the tungsten-free filtrate, as $Mg_2P_2O_7$. This method is open to several criticisms. First, the benzidine tungstate must be precipitated in acid solution, and the presence of acid will tend to re-form the complex acid from the sodium phosphate and tungstate, these being partly split again upon making alkaline for the phosphate precipitation; secondly, as von Knorre's experiments show, a small amount of phosphate is precipitated along with the benzidine tungstate, making reprecipitation necessary to free it completely from phosphate.

Wu's method [1920]. The essential stages for estimation of P_2O_5 are hot sodium hydroxide, precipitation as magnesium ammonium phosphate, ignition, digestion with HCl and reprecipitation of phosphate; for WO_3, ignition and determination of P_2O_5 in residue. Since P_2O_5 was found to be partly volatile, it is not clear why no P_2O_5 is lost in the first process.

This method was tried with the exception that the precipitations of magnesium ammonium phosphate were made in hot solutions. Table II shows some results with control mixtures of phosphate and tungstate[1]. The amount of phosphate (calculated as $Mg_2P_2O_7$) after subtraction of adherent sodium tungstate was not equal to the phosphate originally taken. After the digestion stage, the amount recovered however was in good agreement.

The incomplete removal of tungstic oxide by the digesting process in our hands is shown in the first experiment in Table II and a complete phosphate analysis according to Wu is shown in the second experiment of the same table.

Table II.

Na_2WO_4, $2H_2O$ g.	KH_2PO_4 g.	$Mg_2P_2O_7$ on 1st precipitation g.	Error in 1st precipitation %	WO_3 remaining after HCl g.	Corrected error %	$Mg_2P_2O_7$ on 2nd precipitation g.	Error in 2nd precipitation %
1·5641	0·0902	0·0876	18·7	0·0015	16·8	—	—
0·7833	0·1315	0·1214	12·9	0·0033	8·9	0·1067	0·8

The methods of separation of these mixtures proved so tedious and uncertain that a trial was made of ignition.

The ignition method. It has been stated [Rosenheim and Jaenicke, 1917] that upon heating to a dull red heat, these phosphotungstic acids give off most of their water, leaving behind only metaphosphoric acid and tungstic oxide. The latter should not volatilise until about 1400°, whereas the former should be volatilised by about 800°. By heating the phosphotungstic acid first at 540° to constant weight and then at 1000°, for the best samples practically theoretical figures could be obtained for water and tungstic oxide.

The acid was heated in a crucible supported on a small silica stand in an electric furnace; the temperature was regulated by a variable resistance and was read off directly on a calibrated thermocouple. Heating for some days at

[1] Wu did not publish any figures for controls.

540° and 1000° to drive off water and phosphorus compounds respectively was required. The process however needs little attention. Table III shows the results. There is some uncertainty as to the exact temperature of volatilisation of metaphosphoric acid, and it seems to depend to some extent upon conditions. We have therefore calculated the results expected in two ways, giving respectively the theoretical figures upon assumption that (*a*) the whole of the water disappears at 540°, leaving behind P_2O_5, and that (*b*) one molecule remains, forming metaphosphoric acid.

Table III.

Acid taken g.	H_2O lost at 540° g.	H_2O %	WO_3 left at 1000° g.	WO_3 %
2·3431	0·3864	16·48	2·0995	81·54
1·2880	0·2101	16·31	—	—
3·6913	0·6071	16·45	2·9908	81·02
2·1354	0·3466	16·24	1·7342	81·21
	Average	16·37		81·38

Formula for 1:24-acid: $3H_2O.P_2O_5.24WO_3.60H_2O$.
(*a*), (*b*) Calculated for $60H_2O+3H_2O$ on assumption (*a*) that all H_2O volatilises at 540° and (*b*) that 1 molecule H_2O remains with P_2O_5.

	Observed	Calculated (*a*)	Calculated (*b*)
%	%	%	%
H_2O	16·37	16·56	16·31
WO_3	81·38	81·40	81·34

The figures are so close to the theoretical that there can be little doubt that the method is of value for the 1 : 24-acid. Curiously enough it proved impossible to control it by heating mixtures of sodium tungstate and phosphate; the conditions of volatilisation seemed to be altered. One experiment upon the 1 : 18-acid suggested that it might be also of value for this acid, but it seemed to take longer to attain constant weight.

Conclusion.

The purity of 1 : 24-phosphotungstic acid can be checked by heating successively to constant weight at 540° and 1000°.

PART II. THE INFLUENCE OF HYDRION CONCENTRATION AND SOME OTHER FACTORS ON THE PRECIPITATION OF BASES

(By H. BARNES and R. A. PETERS).

We have studied the influence of concentration of base and of phosphotungstate together with salts upon the initial precipitation. Most work has been done with creatinine, rather less with histidine, and least with a few other bases. More attention has been paid to the 1 : 24-acid, whose greater stability makes it a more useful reagent.

Methods.

Initial point of precipitation. An aqueous solution of the base (1·0 cc.) was treated with 0·1 cc. Na phosphotungstate solution (p_H 6·0). Acid (usually sulphuric) of convenient strength was added from a graduated pipette until a faint permanent cloud was observed. Observation of the initial point was aided by the use of a point source of light in a dark room, using a comparison tube. The sharpness of this point varied with the substance, being very definite with histidine. With creatinine it was much influenced by the speed of addition of the acid (see below).

p_H *range of precipitation.* The base was dissolved in 5·0 cc. water and divided into 1·0 cc. samples (Ostwald pipette). After adding 0·1 cc. of phosphotungstate (90 %) to each, and the requisite sulphuric acid of appropriate strength, the tubes were centrifuged after standing for half an hour. The liquid was used for the determination of p_H and the residue washed, dried and weighed.

Hydrion concentration. As the hydrogen and quinhydrone electrodes were inapplicable with the unstable phosphotungstates, observations were made colorimetrically, or where necessary with the glass electrode. In the latter case duplicate observations agreed to within 0·03 p_H.

Chemical substances.

Creatinine. Commercial samples were purified (1) by way of the zinc chloride compound and (2) by repeated purification from acetone [Edgar and Hinegardner, 1923]. A theoretical value was obtained for nitrogen.

Histidine. More than one sample was used, some from commercial sources, and others prepared in the laboratory by the methods of Raistrick [see Cole, 1926], and of Vickery and Leavenworth [1928]. The melting-points of the compounds varied, but no essential difference was found in the precipitation behaviour. A pure specimen of the dihydrochloride melting at 242° gave the same results as others.

Guanidine sulphate. Recrystallised from commercial sources.

Aminoguanidine sulphate, M.P. 207°. The specimens of *as*-dimethylguanidine and of arginine nitrate were from commercial sources and of doubtful purity.

Phosphotungstate solutions.

An aqueous solution of the phosphotungstic acid was neutralised with NaOH to p_H 6·0 approx., usually in presence of a trace of bromocresol purple. For weaker concentrations 10 % NaOH is sufficient. For the 90 % solutions, which are nearly saturated, it is best to dissolve in the minimum amount of water and add slowly 20 % NaOH cooling the solution between each addition. The use of stronger NaOH or taking to p_H 7·5, or allowing the solution to get hot is apt to cause decomposition. The weaker solutions appeared to keep indefinitely at room temperature, but the stronger ones often deposited crystals after standing. It is not known whether these have a different composition.

Creatinine.

This base was chosen because it was relatively easy to obtain, and had an initial precipitation point lying between p_H 5·0 and 6·0. This point was investigated with amounts of phosphotungstate (*a*) much less than and (*b*) more than were required to precipitate amounts of dissociated creatinine up to 8·0 mg. per cc. (0·0707 *M*). For (*a*) 0·1 cc. of 10 % phosphotungstate was added to 1·0 cc. solution containing the base, and for (*b*) 0·1 cc. of 90 %. As the amounts of acid needed to start precipitation varied, the concentra-

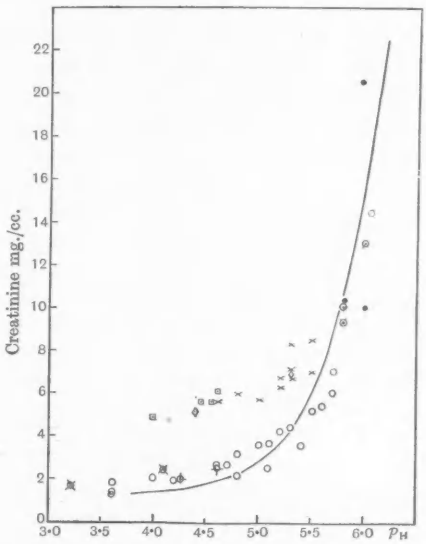


Fig. 1. Variation of initial point of precipitation of creatinine with concentration. Concentrated phosphotungstate solution (1:24). Ordinates creatinine concentration mg./cc., 11·3 mg./cc. = 0·1 *M*. Abscissae p_H.

⊙ × Points at 20°. ◈ 10°. ⊡ 31°. ⊠ Na_2SO_4 added. ⊕ NaCl added.

tions of phosphotungstate at the moment of precipitation might be as an extreme 4·5–8·0 %, and one-tenth of this respectively. With (*b*) points above 7–8 mg./per cc. creatinine, were well defined and could be obtained to within 0·1 p_H. Below this the conditions of precipitation became very important. The lowest points in Fig. 1 (circles) were obtained by one observer working to standard conditions, and represent an extreme. Other points (crosses) are observations of others. When the acid is added very slowly, there is no question that the initial point tends to be pushed to the acid side. The area containing the points is really an indeterminate zone. In (*a*) (Fig. 2) the same phenomena are to be seen. The weaker concentration of phosphotungstate is associated

with a shift of approximately 0·6 p_H for 10–20 mg. creatinine concentration. A few points are included with the 1 : 18-acid.

The results with the 10 % phosphotungstate can be compared directly with those previously published for the commercial acid [Peters, 1930] and are seen to be considerably further to the alkaline side. Commercial acid p_H 2·5, 2·0 mg./cc.; p_H 4·0, 10 mg./cc., and for 1 : 24-acid p_H 4·0 and 5·8; for 1 : 18-acid the values are p_H 5·2 and 6·2. With the 1 : 24-acid above 6·0 mg./cc. creatinine, the initial point becomes largely independent of concentration of base. We have included in the diagram points found in the presence of added sodium chloride and sulphate up to 2·5 % concentration

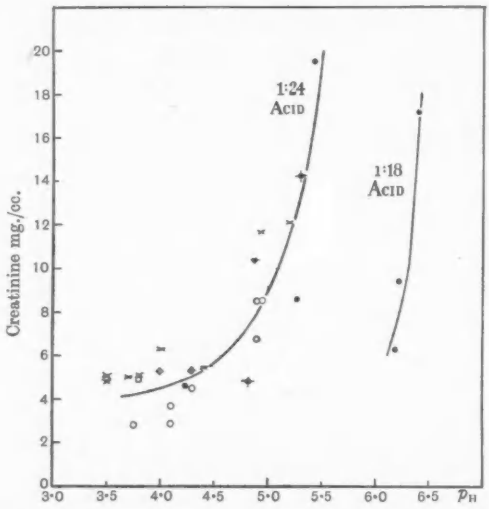


Fig. 2. Variation of initial point of precipitation of creatinine with concentration, for 1:24-phosphotungstate (dilute) and 1:18-phosphotungstate (dilute 10 %). Data as Fig. 1.

and also variations of temperature of $\pm$ 10°. The differences so introduced were slight if at all existent, and were negligible so far as these experiments were concerned. Nor did it make a significant difference if HCl were substituted for H_2SO_4. That traces of creatine could not influence results was also shown upon two occasions by adding creatine; this effectually controlled the possibility of traces of creatine in the creatinine or formed in solution.

Estimation of the p_H range of precipitation showed that with a concentration of 5 mg./cc. base most of the precipitate appeared upon titrating from p_H 6·0 to 2·8; hence the practical issue is that if we collect all the phosphotungstate precipitated between p_H 6·0 and 2·5, we shall have all the creatinine except that which is soluble, some 1·6 mg./cc. or rather more depending upon the concentration of phosphotungstate used.

From the theoretical standpoint, the important general points seem to be (1) the constancy of the initial precipitation point at about p_H 6·1 for both acids when sufficient phosphotungstate is present, (2) the fact that the zone of precipitation extends from p_H 6·0 to 2·0 approximately, over 4·0 units of p_H. The special points are (3) the tendency for the initial precipitation point to shift to the acid side in the case of the 1 : 24-acid with decreasing concentration of phosphotungstate, and (4) the tendency for precipitation to take place more definitely near the initial point with the 1 : 18-salt. Dealing first with the general points, the p_K for creatinine has been shown by Cannan and Shore [1928] to be 4·91 at 15°. The precipitation is therefore roughly co-extensive with the range of dissociation of creatinine. This suggests that it is related to this dissociation. Now the complex precipitated might be either a salt-like compound with phosphotungstate, or some complex with the phosphotungstate molecule as a whole. We could exclude the formation of a salt-like compound upon passing from an acid to an alkaline p_H, if it happened that the dissociation of the phosphotungstic acid was complete at p_H 2·0. It would then be clear that a precipitate formed at p_H 5·0 must consist of a complex of Na phosphotungstate together with dissociated creatinine. In the absence of data in the literature, the dissociation curve of the complex acids has been briefly investigated to settle this point.

Dissociation curves of the phosphotungstic acids. The dissociation curve for the 1 : 24-acid as determined by titration is given in Fig. 3. Up to p_H 7·0 some 19 g.-mol. Na are taken up per 1·0 g.-mol. of the acid and at approximately p_H 7·8 24 g.-mol. Na, or an amount of Na corresponding to each unit of WO_3. It is curious and suggestive that the sudden change in the character of the dissociation curve starts at p_H 7·0 and becomes intense at p_H 7·8, when 24 acidic groups have been neutralised. The points are here apt to become uncertain, and there is evidence of change of equilibrium, because some time must be allowed to elapse after addition of alkali before the final p_H is reached; immediately after titration the p_H is much more alkaline than the equilibrium point. Further investigation is needed, but the above is sufficient to show that the phosphotungstic acid molecule must be regarded as a polyacidic micelle; the dissociation curves of the different acidic centres overlap to form an almost continuous straight line over the range p_H 2·0–7·0. The behaviour is rather analogous to that of a protein, though uncomplicated by basic dissociations. Since gradual neutralisation of the acid occurs over the whole range p_H 2·0–7·0, we cannot exclude the possibility that salt-like compounds between dissociated phosphotungstic acid and base contribute to the precipitate formation. Two kinds of compounds can be pictured, if we assume that no actual change is taking place in the phosphotungstate molecule itself, (1) some molecules form creatinine phosphotungstates, while others remain uncombined; (2) each phosphotungstate molecule acts as a polyacidic micelle, and takes up a certain proportion of base. Though the data do not admit of decision, we think that (2) is more likely. This would give precipitates, con-

sisting of Na-creatinine-phosphotungstate with some undissociated groups in the molecule according to the p_H.

The dissociation curve for the 1 : 18-acid was briefly investigated and proved to be different. Our supplies were limited and did not admit of detailed study, but there seemed to be no buffering action between p_H 2·0 and 7·0. The difference provides one avenue to explore in explaining the variations in precipitation behaviour between the two acids.

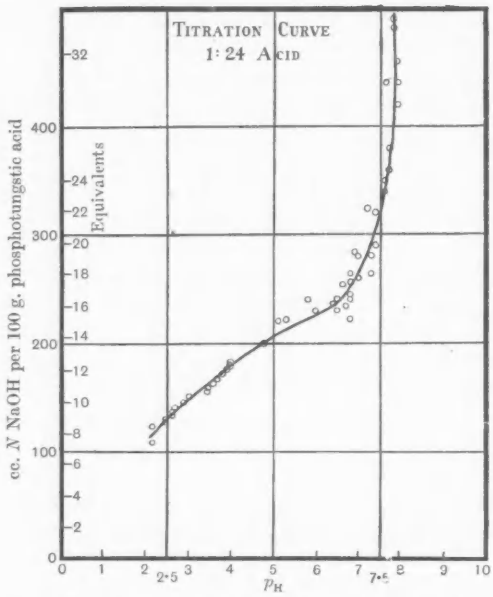


Fig. 3. Titration curve 1:24-acid. *M* equivalents NaOH.

Diagrammatically we should have

undissociated groups (*n*)
Phosphotungstic acid—organic base (creatinine) (*m*)
Na (*p*)

Here *n*, *m*, *p* represent whole numbers, the values of which vary according to the p_H and the concentration of phosphotungstate and Na.

Composition of the precipitates.

We have tested whether the precipitates contain sodium and found this present. They are clearly different from those formed by bases in acid solution. For instance according to Drummond [1918] 1 g. of creatinine gives in acid solution less than 10 g. phosphotungstate. In our case, 1 g. often gave 12–13 g. Experiments upon the relative proportion of Na and creatinine have been

made as follows. Approximately 100 mg. samples of creatinine have been treated with 1·5 cc. H_2O, 1·5–2·0 cc. 90 % Na phosphotungstate (p_H 6·0) added, and the p_H adjusted to 5·0 and 3·0 approximately. After standing for one hour or more, the precipitates were removed by centrifuge and the precipitate washed once with 1·0 cc. H_2O containing a trace of phosphotungstate at the p_H used. If taken within 1 hour, precipitation has not reached completion. They were not dried, owing to the possibility of introducing changes in composition and variations in the usual practical procedure. They were extracted as in the vitamin B_1 work by grinding with baryta and H_2O 4 times. The resulting solution was acidified with H_2SO_4 to Congo red, heated and made up to a known volume after filtration. One-fifth was taken for the estimation of creatinine by Folin's method, and the remainder evaporated to dryness and incinerated; the resulting Na_2SO_4 was determined by drying to constant weight in a crucible. Controls, in which known amounts of NaOH and creatinine were added to phosphotungstate at a given p_H and the whole was extracted with baryta, gave a recovery of 95–98 % for creatinine but not more than 75 % for sodium.

The experiments, some 10 in all, showed that Na was always found to be present in the baryta extracts, but the ratio creatinine/Na varied rather widely from the extremes of 10/1 to 4/1. The proportion of Na in the precipitates appeared to decrease when the precipitates were allowed to stand for periods over 1 hour. The fact that the weight of precipitate is some 12–13 times that of the creatinine taken is most simply explained upon the view that the precipitates contain on the average 5 molecules creatinine per molecule of phosphotungstic acid. Upon the assumption that creatinine is here monobasic, one molecule attached at one acidic grouping, using data from the dissociation curve, we may calculate that approximately at p_H 3·0 the ratio of creatinine/Na should be 4·7 and 3·4 at p_H 5·0. Incomplete recovery of Na will not explain the differences from the observed ratios, so that we are faced with the conclusion that upon balance each creatinine molecule displaces more than one Na. At p_H 5·0 replacement of 2Na would lead to a ratio of 12/1 and at p_H 3·0 a higher ratio still. The increase in the proportion of creatinine/Na upon standing must be due either to a continued replacement of Na in the precipitate by creatinine (a kind of base exchange like that of Ca in fuller's earth [compare Phelps, 1931]), or to the formation of phosphotungstates of more than one kind. In several cases crystals formed, so that the compounds must be well defined. Their evident complexity makes caution advisable in framing hypotheses.

If however the precipitation is due to dissociated creatinine, as seems most probable, the amount dissociated at a given p_H is known from the usual relation[1] $p_k + \log \frac{a}{1-a} = p_H$, where $p_k = 4{\cdot}91$. We can calculate for any p_H the total concentration needed to give an amount of dissociated creatinine equal

[1] p_k, apparent dissociation constant; a = ratio $\frac{\text{dissociated acid}}{\text{total acid}}$.

to the known solubility; upon hypothesis this should be proportional to the amount which just forms a precipitate. The curve in Fig. 1 is so calculated for a solubility of dissociated creatinine phosphotungstate of 1·25 mg./cc. The agreement with the points determined is sufficiently close to suggest that the fundamental assumption may be justified. In Fig. 2, a similar curve is drawn for a solubility of 4 mg./cc. In the latter case, there is a suggestion of discontinuity in the determined points, which was supported by the results of an attempt to determine the solubility product relations for low dilutions of phosphotungstate.

The concentration of phosphotungstic acid required to initiate precipitation at a constant p_H.

1·0 cc. solution (Ostwald pipette), containing 20 mg. dry creatinine, was treated with 0·264 cc. *N*/3 HCl unless otherwise stated to bring to p_H 4·90 approx. To this was added from a micro-burette, 4·95 % Na phosphotungstate solution. The p_H was then determined colorimetrically using a comparator. All solutions were stored in bottles protected from air with soda lime tubes to eliminate CO_2 as much as possible. Na phosphotungstate p_H 4·90 was made by neutralising 10 % 1 : 24-phosphotungstic acid in the ratio of 1·02 cc. 0·2 *N* NaOH to 1·0 cc. acid. Table IV A gives some results for immediate precipitation and IV B for precipitation upon standing for 24 hours.

Table IV. *Precipitation of creatinine by sodium phosphotungstate at constant* p_H.

In all cases 1·0 cc. creatinine solution (20 mg. creatinine) used.

0·264 cc. *N*/3 HCl added in Exps. 1, 2, 3, 8, 9, 10.
0·15 cc. " Exps. 4, 6, 7.
0·08 cc. " Exp. 11.
0·14 cc. " Exp. 12.

Exps. 11, 12 with 90 % Na phosphotungstate, the remainder with 4·95 %.

Exp.	cc. phospho-tungstate	p_H	Conc./cc. Crea-tinine (C)	Conc./cc. Phospho-tungstate (P)	C × P	
A. Temp. 18·5±0·5°. Immediate precipitation.						
1	0·605	4·8	10·7	15·6	167	
2	0·385	5·0	12·15	11·6	140	
3	0·300	4·9	12·8	9·5	122	
4	0·144	5·3	15·5	5·51	85	
5	0·150	5·3	15·4	5·71	85	
B. Temp. 25·5±0·5°. Slow precipitation or no precipitate (22 hours).						
6	0·02	5·3	17·1	0·085	1·45	Precipitation
7	0·01	5·3	17·2	0·043	0·7	Very small precipitation
8	0·05	4·9	15·3	0·189	2·9	Precipitation
9	0·035	4·9	15·3	0·133	2·04	Very small precipitation
10	0·02	4·9	15·3	0·077	1·2	No precipitation
11	0·10	6·0	17·0	70·6	1200	No precipitation
12	0·10	5·6	16·1	72·5	1160	Very small precipitation

For immediate precipitation, we have for p_H 4·8–5·0 a product of creatinine and phosphotungstate of 122–167 mg./cc., but at p_H 5·3 a product of 85,

i.e. less phosphotungstate seems to be required to produce an immediate precipitate at p_H 5·3. This suggests that the precipitates are complex, but no emphasis can be placed upon these figures; they are not true equilibrium points, because, upon standing for 24 hours, the precipitation tends to progress and beautiful crystals appear, of at least two different forms, needles and cubic prisms. These merit further study. Provided that the p_H is within the precipitation zone, surprisingly little phosphotungstic acid will suffice to give crystals in 24 hours, amounts far below those giving visible precipitates even within an hour (see Table IV B). It should be emphasised that outside the indeterminate zone, phosphotungstate and creatinine may be kept together in solution almost indefinitely without crystallisation or precipitation (see especially Exp. 11).

As a working hypothesis, it is suggested that the main precipitation is related to the presence of dissociated creatinine. The complete theory will have to take into account as well as the p_H the concentrations of creatinine and phosphotungstate, and probably the equilibrium between more than one form of compound.

Note. There is one possibility, which requires mention. It has been recently shown that at a liquid interface, there is an apparent shift of dissociation constant [Peters, 1931]; similar conclusions follow from work on charcoal adsorption for the solid interface [Phelps and Peters, 1929; Phelps, 1931]. This is likely to happen also with these precipitates, and probably provides a partial explanation of the change in character upon standing.

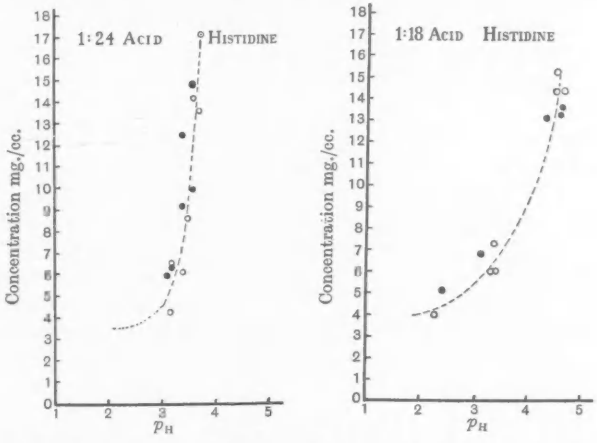


Fig. 4. Variation of initial point of precipitation of histidine (hydrochloride) with concentration. 1:24- and 1:18-acids. Concentration mg./cc., 9·1 mg./cc. = 0·1 *M*.
○ = 10 % acid. ● = 90 % acid.

Histidine.

The curves shown in Fig. 4 have been obtained by the same technique as in the case of creatinine, histidine hydrochloride being used. The curves for the 1 : 24- and 1 : 18-acids have the same position, differing in this from

creatinine. They bear the same relation to the dissociation curve p_H 1·0–4·0 (one of the dissociating groups of histidine) which we find in creatinine, but the same explanation does not apply. There is now much evidence for belief in the zwitterion constitution of the amino-acids [Bjerrum, 1923; Birch and Harris, 1930; Borsook and Wasteneys, 1930]. According to this view the group dissociating over this range is the —COOH grouping. Hence, we cannot take the simple explanation adopted for creatinine; we must believe that for histidine a precipitation of the compound formed over the range of dissociation of the —NH group is inhibited as long as the —COOH group is ionised. The matter could be tested by conductivity determinations. We should of course get precisely the same curve, but the initial point of precipitation would coincide with the dissociation of the —COOH group. This might explain the coincidence of the precipitation curves for the two different acids, because the compounds would already exist in solution at the p_H at which precipitation started.

Other bases.

We have not explored the field of the relation of chemical constitution to p_H of precipitation at all fully, and give the incomplete data in the belief that they may be of value to other workers. Fig. 5 represents the most probable value of the main precipitation point. Some considerable difference is made in some cases by the method of addition. For instance, for aminoguanidine sulphate, a very pure specimen, addition of Na phosphotungstate at p_H 5·0 to a solution containing 11 mg./cc. caused an opalescence. This became definite precipitation at p_H 7·4. The data should be studied in relation to those for the commercial acid. The points for this are seen to lie often intermediately between the very different points given by the 1 : 18- and 1 : 24-acids. With guanidine, as previously indicated, precipitation occurs on the alkaline side in low concentrations for the commercial acid and for the 1 : 24-acid, of which the commercial largely consists. It is more sharply defined for the 1 : 24-acid. Introduction of the amino-group modifies the precipitation, shifting the points to the alkaline side at a low concentration.

This alkaline precipitation by guanidine introduces the last complication which we have met, and seems to need another explanation, as it is related to the dissociation in the opposite sense. If $p_{K'_b}$ for guanidine is 8·5, then this compound only precipitates in low concentration when undissociated, and must be forming a complex. It is probably significant that this precipitation is coincident with the sudden bend in the dissociation curve of the 1 : 24-acid.

We may describe the scope of the theoretical problem by saying that it must be possible to decide from a knowledge of the compound whether it will form phosphotungstates either (*a*) with dissociated basic groups (example creatinine), or (*b*) with dissociated groups which precipitate only when the

influence of other groups ceases to operate (example histidine), or (*c*) complexes with undissociated basic groups, insoluble in alkaline solutions upon the lines of guanidine. A complete solution should enable us to predict much about the constitution of an unknown base precipitable by phosphotungstate from its behaviour to c_H, and conversely the most likely method of selecting a base from some biochemical mixture. Fortunately the complication introduced by guanidine is not important unless precipitation starts at an alkaline p_H. It is

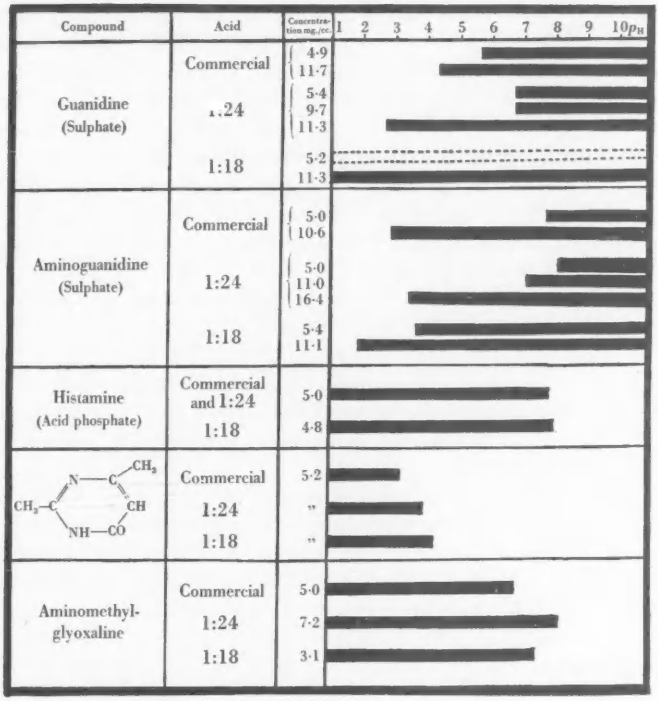


Fig. 5. Zones of precipitation (p_H), marked as black lines, for various compounds with three different phosphotungstic acids.

likely that substances attached to guanidine-like compounds are difficult to free from phosphotungstate with baryta. This may explain the losses sometimes found at a phosphotungstic stage. For instance Kinnersley and Peters [1925] found it to be quite impossible to recover vitamin B_1 from phosphotungstic acid precipitates at an early stage in their work.

The precipitates at acid p_H will usually dissolve at an alkaline p_H, upon addition of NaOH at a point some 2·0 p_H units more alkaline than the initial precipitation point, but there is a tendency to decomposition.

Some practical issues.

The applications of this work to the means of (1) characterising and (2) separating bases may be shortly considered in the light of these facts.

(1) It must now be realised that the original method of precipitating in the presence of minimum amounts of phosphotungstate must be rigorously watched and estimates of p_H of initial precipitation made at concentrations above the indeterminate zone. Solubility of the phosphotungstate matters much less in the case of substances like histidine or those like vitamin B_1 in which the vitamin compound is very insoluble. The latter precipitates at a dilution of less than 1 : 100,000 at p_H 5·0 [Kinnersley, O'Brien and Peters, 1932]. Presumably here the vitamin must precipitate very close to the beginning of its dissociation curve. (2) Several points are important in dealing with separations. The method used by Kinnersley and Peters [1930] for the separation of vitamin B_1, where the phosphotungstate is kept in minimum concentration, gives good results and has the great practical advantage that the amounts of phosphotungstate used are much smaller than the amounts needed to precipitate all the bases. This saves an expensive reagent. Their method, determined empirically, of working at different dilutions and gradually increasing the strength in successive fractionations is theoretically sound. Provided that a base precipitates between p_H 2·0 and 7·0, it should be possible to separate it by the use of suitable values of p_H and varying precipitations at various dilutions. These methods might be combined with fractionation in very acid solution. It is of course essential that the phosphotungstate should be very insoluble.

Precipitates at p_H 2–7 will give Na as well as organic bases upon decomposition. If therefore complete freedom from base is required, such precipitates should be treated with $N/10$ acid to free them from Na before decomposition with baryta. It is interesting to note that these principles of separation have been successfully applied at one stage in the separation of vitamin B_4 [Barnes, O'Brien and Reader, 1932], the vitamin appearing in the precipitate more acid than p_H 3·0.

Summary.

The precipitation of creatinine and of histidine HCl by pure phosphotungstic acids has been studied in detail and some data about the precipitation of certain other bases have been determined. The dissociation curves of the acids have also been investigated, and the data considered to some extent theoretically, but especially in their practical bearing upon newer methods of separating bases by the sodium phosphotungstate technique.

We are indebted to W. Wakelin for skilful assistance and to Dr Gulland for the specimen of aminomethylglyoxaline.

REFERENCES.

Barnes, O'Brien and Reader (1932). *J. Physiol.* **76**, 8 *P.*
Birch and Harris (1930). *Biochem. J.* **24**, 564.
Bjerrum (1923). *Z. physikal. Chem.* **104**, 147.
Borsook and Wasteneys (1930). *J. Gen. Physiol.* **13**, 509.
Brandhorst and Kraut (1888). *Liebig's Ann.* **249**, 373.
Britton and Robinson (1932). *Trans. Faraday Soc.* **28**, 531; *J. Chem. Soc.* 1955.
Cannan and Shore (1928). *Biochem. J.* **22**, 920.
Cole (1926). Practical physiological chemistry, 7 ed. (Heffer, Cambridge.)
Drechsel (1887). *Ber. deutsch. chem. Ges.* **20**, 1452.
Drummond (1918). *Biochem. J.* **12**, 5.
Edgar and Hinegardner (1923). *J. Biol. Chem.* **56**, 881.
Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 109.
Gibbs (1877). *Ber. deutsch. chem. Ges.* **10**, 1384.
—— (1880). *J. Amer. Chem. Soc.* **2**, 220.
Gooch (1879). *J. Amer. Chem. Soc.* **1**, 391.
Kehrmann (1887). *Ber. deutsch. chem. Ges.* **20**, 1805.
—— and Fraenkel (1891). *Ber. deutsch. chem. Ges.* **24**, 2326.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.
—— —— (1930). *Biochem. J.* **24**, 1856.
—— O'Brien and Peters (1932). *J. Physiol.* **76**, 17 *P.*
Péchard (1889). *Compt. Rend. Acad. Sci.* **109**, 301.
Peters (1930). *Biochem. J.* **24**, 1852.
—— (1931). *Proc. Roy. Soc. Lond.* A **133**, 140.
Phelps (1931). *Proc. Roy. Soc. Lond.* A **133**, 17.
—— and Peters (1929). *Proc. Roy. Soc. Lond.* A **124**, 554.
Rosenheim and Jaenicke (1917). *Z. anorg. Chem.* **101**, 215.
Scheibler (1872). *Ber. deutsch. chem. Ges.* **5**, 801.
Sprenger (1880). *J. prakt. Chem.* (2) **22**, 418.
Vickery and Leavenworth (1928). *J. Biol. Chem.* **78**, 627.
von Knorre (1908). *Z. anal. Chem.* **47**, 37.
Wu (1920). *J. Biol. Chem* **43**, 189.

INDEX

ABBASSI, M. A. A simple method for the determination of the melting-point of fats and allied substances 1857
Acacatechin, supposed production of maclurin from (Healey) 388
Acetic acid, effect of, on deposition of liver-glycogen (Ponsford and Smedley-MacLean) 1340
Acetone bodies, Van Slyke's method of determination of, applied to small volumes of blood and urine (Nanavutty) 1391
Acidosis, nephritic, electrolyte distribution and acid-base equilibrium in the serum in cases of (Greene, Wakefield, Power and Keith) 1377
Acipenser sturio, *see* Sturgeon
ADAIR, G. S., *see* ROCHE, J.
ADAIR, M. E., *see* ROCHE, J.
ADAM, N. K., DANIELLI, J. F., HASLEWOOD, G. A. D. and MARRIAN, G. F. Surface films of oestrin derivatives 1233
Adrenaline, colorimetric determination of, in suprarenal gland extracts (Barker, Eastland and Evers) 2129
Adrenaline injections, effect of, on hexosemonophosphate content of rat muscle (Bell) 1601
Alcohol, effect of, on oxygen uptake of brain tissue (Robertson and Stewart) 65
Aldehydes, determination of (Clift and Cook) 1788
ALLCROFT, W. M., *see* GODDEN, W.
ALLOTT, E. N., DAUPHINEE, J. A. and HURTLEY, W. H. The determination of small quantities of iodine in blood 1665
Aluminium, biochemistry of (Mackenzie) 833
Aluminium, effect of, on growth and reproduction in the rat (Mackenzie) 833
Aluminium, intestinal absorption of (Mackenzie) 833
Aluminium, occurrence of, in the thyroid (Mackenzie) 833
Amino-acid absorption, relationship of, to protein synthesis in the mammary gland (Blackwood) 772
Amino-acid, effect of administration of, on blood-urea in abnormal mental states (Lockwood and Davies) 745
Amino-acids, basic, determination of, in small amounts of protein (Rosedale and da Silva) 369
Amino-acids, basic, of egg-albumin (Vickery and Shore) 1101
Amylase, influence of guanidine and derivatives on the action of (Mystkowski) 910
Amylase of germinated barley, effect of starch concentration upon rate of hydrolysis by (Hanes) 1406
Amylase—starch—protein systems, effect of salts on (Baumgarten) 539
Anaemia, pernicious, glutathione content of substances used in the treatment of (Fleming) 461
ANDRZEJEWSKI, H. Effect of addition of salts on the isoelectric point of proteins. III 1397
Anthocyanins (Robinson and Robinson) 1647
Antibacterial substance of Fleming, produced from glucose by *P. chrysogenum* (Clutterbuck, Lovell and Raistrick) 1907
Antigrowth factor in extracts of parathyroid glands (Eastland, Evers and Thompson) 2123
Antimony trichloride test for vitamin A, interpretation of the colour match in (Morgan) 377
Antiscorbutic activity of cortex of ox suprarenal (Harris and Ray) 2067; (Zilva) 2182
Antiscorbutic activity of lemon juice, non-specificity of reducing power as a measure of (Zilva) 1624
Antiscorbutic fraction of lemon juice. X (Johnson and Zilva) 871
Antiscorbutic potency of apples (Crane and Zilva) 2177
Antiscorbutic value of fresh lime juice (Hassan and Basili) 1846
Antiscorbutic, *see also* Vitamin C
Antiseptics, related to indan-1:3-dione (Robinson, Suthers and Walker) 1890
Apples, antiscorbutic potency of (Crane and Zilva) 2177
Arginine, determination of, by the Sakaguchi reaction (Jorpes and Thorén) 1504
Arginine, effect of administration of, on the excretion of creatinine (Shapiro and Zwarenstein) 1880
Armillaria mellea, action of, on wood (Campbell) 1829
ARMITAGE, E., PICKERING, J. W. and MATHUR, S. N. The inception of blood clotting 853
ARMSTRONG, A. R. and WALKER, E. The bromine reaction of pregnancy urine 143
ARMSTRONG, A. R., *see also* HARDING, V. J.
ASKEW, F. A., BOURDILLON, R. B. and WEBSTER, T. A. The production of vitamin D in a glow discharge 814
Asparagine, estimation of glutamine in presence of (Chibnall and Westall) 122
Asparagine, isolation of, from an enzymic digest of edestin (Damodaran) 235
Aspergillus niger, action of, on sodium hexosediphosphate in presence of toluene (Suthers and Walker) 317
Aspergillus versicolor, growth of, on higher paraffins (Hopkins and Chibnall) 133
Autoxidation of fatty acids (Ellis) 791
AYYADI, A. S. EL., *see* HASSAN, A.

Bacillus, specific carbohydrate of the tubercle (Gough) 248
Bacteria, effect of associated growth on lactic acid production by certain (Tatum, Peterson and Fred) 846
Bacterial enzymes liberating molecular hydrogen (Stephenson and Stickland) 712
Bacterial gelatinase, influence of the medium on production of (Haines) 323
Bacterial metabolism (Hewitt) 208, 464

BAILEY, K. and NORRIS, F. W. The nature and composition of the mucilage of the seed of white mustard (*Brassica alba*) 1609
BANKS, A. and HILDITCH, T. P. The body fats of the pig. II. Some aspects of the formation of animal depôt fats suggested by the composition of their glycerides and fatty acids 298
*iso*Barbituric acid, effect of, on excretion of neutral sulphur and ethereal sulphate by the dog (Lawrie and Price) 622
Barium, titrimetric method for the microdetermination of (King) 586
BARKER, J. H., EASTLAND, C. J. and EVERS, N. The colorimetric determination of adrenaline in suprarenal gland extracts 2129
BARNES, H., O'BRIEN, J. R. P. and READER, V. Vitamin B_6 2035
BARNES, H. and PETERS, R. A. The use of pure phosphotungstic acids in the precipitation of bases. I. Method of checking the purity of 1 : 24-phosphotungstic acid. II. The influence of hydrion concentration and some other factors on the precipitation of bases 2203
BARRETT, J. F. and JONES, E. B. A colorimetric method for the direct determination of urea in urine 1246
Bases, precipitation of, influence of hydrogen ion concentration and other factors on (Barnes and Peters) 2203
BASHA, M. K. A., *see* HASSAN, A.
Basic amino-acids, determination of, in small quantities of protein (Rosedale and da Silva) 369
BASILI, R., *see* HASSAN, A.
BASSINDALE, R., *see* SOUTHGATE, B. A.
BAUMGARTEN, G. Structure and enzyme reactions. XI. Action of salts in the systems: starch—amylase—proteins 539
BEHRE, J. A. A note on the determination of ergothioneine in blood-filtrates 458
BELL, D. J. The absence of abnormal forms of glucose from blood dialysates 349
BELL, D. J. Wood cellulose. I. Methylation of the α-cellulose 590
BELL, D. J. Wood cellulose. II. The depolymerised celluloses of wood and cotton 598
BELL, D. J. Wood cellulose. III. The "resistant portions" 609
BELL, D. J. Observations on the hexosemonophosphate content of mammalian muscle. I. Experiments on rats injected with adrenaline 1601
BELL, D. J. Wood cellulose. IV. Further investigation of the "resistant portion" of Thiriena pulp 1772
BELL, G. H. and PATERSON, A. R. C. A p_H chart 454
Benzidine reaction of blood, factors influencing, as applied to determination of haemoglobin (McFarlane and Hamilton) 1050
Benzidine test, simplified, for blood in clinical material (Ingham) 1124
BERNAL, J. D., *see* PIRIE, N. W.
BIERRING, E. and NIELSEN, E. The composition of the tissues of albino rats treated with alkaline anterior pituitary extracts 1015
Bile pigment, isolation of, with direct van den Bergh reaction (Griffiths) 1155
Bilirubin and the van den Bergh reaction (Fowweather) 165
BIRKINSHAW, J. H. and RAISTRICK, H. Studies in the biochemistry of micro-organisms. XXIII. Puberulic acid, $C_8H_6O_6$ and an acid $C_8H_4O_6$, new products of the metabolism of glucose by *Penicillium puberulum* Bainier and *Penicillium aurantio-virens* Biourge. With an appendix on certain dihydroxybenzenedicarboxylic acids 441
BLACKWOOD, J. H. The absorption of milk precursors by the mammary gland. III. The relation of amino-acid absorption to protein synthesis 772
BLACKWOOD, J. H. and STIRLING, J. D. An iodimetric method for the determination of iron in blood 353
BLACKWOOD, J. H. and STIRLING, J. D. The absorption of milk precursors by the mammary gland. I. The Kaufmann-Magne technique 357
BLACKWOOD, J. H. and STIRLING, J. D. The absorption of milk precursors by the mammary gland. II. The relation of blood-sugar absorption to lactose secretion 362
BLACKWOOD, J. H. and STIRLING, J. D. The absorption of milk precursors by the mammary gland. IV. Aspects of the phosphorus metabolism of the mammary gland 778
BLACKWOOD, J. H. and STIRLING, J. D. The absorption of milk precursors by the mammary gland. V. Physico-chemical aspects of milk secretion 1127
Blood, action of bone phosphatase on phosphoric esters of (King) 1697
Blood clotting, inception of (Armitage, Pickering and Mathur) 853
Blood, cow's, changes in composition of, at time of calving, and comparison of, with that of calf (Godden and Allcroft) 1640
Blood, determination of acetone bodies in small volumes of (Nanavutty) 1391
Blood, determination of small quantities of iodine in (Allott, Dauphinee and Hurtley) 1665
Blood-dialysates, absence of abnormal forms of glucose from (Bell) 349
Blood-filtrates, determination of ergothioneine in (Behre) 458
Blood-filtrates, influence of glutathione on reduction and rotation of (Groen and Meyer) 624
Blood, hyperglycaemic, of diabetics without glycosuria, reducing substances of (Rabinowitch) 963
Blood, iodimetric determination of iron in (Blackwood and Stirling) 353
Blood, isolation of ergothioneine from (Williamson and Meldrum) 815
Blood-serum in the rabbit, variations in the calcium of (Bourne and Campbell) 183
Blood, sheep's, reciprocal relationship of calcium and inorganic phosphorus of (Fraser) 2166
Blood, simplified benzidine test for, in urine and other clinical material (Ingham) 1124
Blood-sugar absorption, relation of, to lactose secretion (Blackwood and Stirling) 362
Blood, sugar in, and lactic acid in brain, relation of (Holmes and Sherif) 381

Blood-urea changes in abnormal mental states after administration of amino-acid (Lockwood and Davies) 745
Blood, volatile chlorine of (Morris and Morris) 2015
Boas Fixsen, M. A., *see* Fixsen, M. A. B.
Bone analysis and radiography, comparison of, in the estimation of vitamin D (Bourdillon and Bruce) 506
Bones of rats on rachitogenic diet, relation of graded doses of vitamin D to ash content of (Hume, Pickersgill and Gaffikin) 488
Bourdillon, R. B. and Bruce, H. M. The determination of vitamin D. II. A comparison of radiography and bone analysis in the estimation of vitamin D 506
Bourdillon, R. B., Bruce, H. M. and Webster, T. A. The determination of vitamin D. III. The stability of preparations of vitamin D 522
Bourdillon, R. B., *see also* Askew, F. A.
Bourne, M. C. and Campbell, D. A. Variations of serum-calcium in the rabbit 183
Boyland, E. The precipitation and protection of vanadic acid by serum-proteins 1383
Brain, oxidations by the (Quastel and Wheatley) 725
Brain, relation of lactic acid in, to sugar in blood (Holmes and Sherif) 381
Brain tissue, effect of alcohol on oxygen uptake of (Robertson and Stewart) 65
Brain tissue, effects of narcotics on dehydrogenations by (Davies and Quastel) 1672
Brain tissue, p_H of (Holmes) 2010
Brassica alba, *see* Mustard, white
Bromine reaction of pregnancy urine (Armstrong and Walker) 143
Brown, A. M., *see* Smith, M. L.
Bruce, H. M., *see* Bourdillon, R. B.
Brussels sprout, wax metabolism in the leaves of (Sahai and Chibnall) 403
Buston, H. W. and Nanji, H. R. The preparation of methyl esters of pectic acid 2090
Buxton, P. A. The proportion of skeletal tissue in insects 829

Cabbage, calcium content of (Cowell) 1422
Calcium and inorganic phosphorus of sheep's blood, reciprocal relationship of (Fraser) 2166
Calcium and magnesium, ultra-filtration of, from human sera (Watchorn and McCance) 54
Calcium content of cabbage (Cowell) 1422
Calcium, variations of, in the blood-serum of the rabbit (Bourne and Campbell) 183
Calf, comparison of blood of, with that of dam (Godden and Allcroft) 1640
Cambden, M. R., *see* Coward, K. H.
Campbell, D. A., *see* Bourne, M. C.
Campbell, W. G. The chemistry of the white rots of wood. III. The effect on wood substance of *Ganoderma applanatum* (Pers.) Pat., *Fomes fomentarius* (Linn.) Fr., *Polyporus adustus* (Willd.) Fr., *Pleurotus ostreatus* (Jacq.) Fr., *Armillaria mellea* (Vahl.) Fr., *Trametes pini* (Brot.) Fr., and *Polystictus abietinus* (Dicks.) Fr. 1829
Cancer, hyperglycaemia of (Woodward and Fry) 889
Capillary activity of lecithin-cholesterol dispersions (Jones and Lewis) 633
Capillary behaviour of glycine (Jones and Lewis) 638
Carbohydrate-free diet, metabolism of animals on a (Hynd and Rotter) 578
Carbohydrate metabolism after starvation (Goldblatt and Ellis) 991
Carbohydrate metabolism in human trypanosomiasis (Wormall) 1777
Carbohydrate, specific, of the tubercle bacillus (Gough) 248
Carbohydrates and lactic acid of haddock muscle after death (Macpherson) 80
Carbon dioxide retention of serum, use of, in measurement of tissue glycolysis (Dickens and Šimer) 90
Carotene, conversion of, into vitamin A in the cow (Moore) 1
Carotene, isomerisation of, by antimony trichloride (Gillam, Heilbron, Morton and Drummond) 1174
Case, E. M. The determination of pyruvic acid 753
Case, E. M. The origin of pyruvic acid in muscle 759
Caseinogen, hydrolysis of, by pepsin and by trypsin-kinase (Stirling and Wishart) 1989
Castrated male rats, vitamin A deficiency in (Sampson and Korenchevsky) 1542
Castration, urinary creatinine after (Schrire and Zwarenstein) 118
Catalytic oxidation of uric acid (Dobrowolska) 543
Cellulase from symbiotic intestinal flagellates of termites and the roach, *C. punctulatus* (Trager) 1762
Cellulose, cotton (Bell) 598
Cellulose, determination of, in soil (Daji) 1275
Cellulose, wood (Bell) 590, 598, 609, 1772
Cerebrospinal fluid, inorganic constituents of. (Watchorn and McCance) 54
Channon, H. J. and Saby, M. K. El. Fat metabolism of the herring 2021
Chemistry of cooking (Sinoda and Kodera) 650
Chibnall, A. C. and Westall, R. G. The estimation of glutamine in the presence of asparagine 122
Chibnall, A. C., *see also* Damodaran, M., Hopkins, S. J., Smith, J. A. B., Miller, E. J., *and* Sahai, P. N.
Chick, H. and Jackson, H. M. Note on the International Standard for the antineuritic vitamin B_1 1223
Cheese-ripening studies (Sadler, Eagles and Pendray) 1532
Choleic acids (Sobotka and Goldberg) 555; (Sobotka and Kahn) 898; (Sobotka and Goldberg) 905
Choleic acids; keto-enol tautomerism and co-ordination compounds (Sobotka and Kahn) 898
Choleic acids, separation of optical antipodes of, by means of co-ordination compounds (Sobotka and Goldberg) 905
Choline-esterase (Stedman, Stedman and Easson) 2056

CLIFT, F. P. and COOK, R. P. A method of determination of some biologically important aldehydes and ketones, with special reference to pyruvic acid and methylglyoxal 1788
CLIFT, F. P. and COOK, R. P. The 2 : 4-dinitrophenylhydrazones of some ketonic acids of biological importance 1800
CLIFT, F. P. and COOK, R. P. Triose dehydrogenase. I 1804
Clotting, blood, inception of (Armitage, Pickering and Mathur) 853
CLUTTERBUCK, P. W., LOVELL, R. and RAISTRICK, H. Studies in the biochemistry of micro-organisms. XXVI. The formation from glucose by members of the *Penicillium chrysogenum* series of a pigment, an alkali-soluble protein and penicillin—the antibacterial substance of Fleming 1907
CLUTTERBUCK, P. W., OXFORD, A. E., RAISTRICK, H. and SMITH, G. Studies in the biochemistry of micro-organisms. XXIV. The metabolic products of the *Penicillium brevi-compactum* series 1441
Cocksfoot grass, glyceride fatty acids of (Smith and Chibnall) 218
Cocksfoot grass, phosphatides of (Smith and Chibnall) 1345
Cod-liver oil, determination of vitamin A in, by measurement of absorption in the ultra-violet (Coward, Dyer and Morton) 1593
Cod-liver oil, unsaponifiable fraction of (Weidemann) 264
Co-enzyme for urease, question of (Sumner and Kirk) 551
Collagen fibre-bundles, single, swelling of (Marriott) 46
Colloid, amphoteric, experimental demonstration of movements and precipitations of, in electric field (Penfold and Sutherland) 1839
COOK, R. P., *see* CLIFT, F. P.
Cooking, chemistry of (Sinoda and Kodera) 650
Co-ordination compounds, choleic acid, keto-enol tautomerism of (Sobotka and Kahn) 898
Co-ordination compounds, choleic acid, separation of optical antipodes by means of (Sobotka and Goldberg) 905
Copper, microcolorimetric determination of, in organic substances (McFarlane) 1022
Cotton cellulose (Bell) 598
Cow, changes in the composition of blood of, at time of calving, and comparison of blood of, with that of calf (Godden and Allcroft) 1640
COWARD, K. H. Variation in growth response of rats in vitamin A tests compared with the variation in rats growing normally 691
COWARD, K. H., CAMBDEN, M. R. and LEE, E. M. A stock diet for rats bred for vitamin tests 679
COWARD, K. H., DYER, F. J. and MORTON, R. A. The determination of vitamin A in cod-liver oils (*a*) biologically, (*b*) chemically, (*c*) physically, with a statistical examination of the results. II. Further evidence that the intensity of absorption at 328 $m\mu$ gives the best agreement with the biological measure of vitamin A in cod-liver oils 1593
COWARD, K. H., KEY, K. M. and MORGAN, B. G. E. The quantitative determination of vitamin D by means of its growth-promoting property 1585
COWELL, S. J. A note on the calcium content of cabbage 1422
CRANE, M. B. and ZILVA, S. S. The antiscorbutic potency of apples. V 2177
Creatinine, effect of administration of arginine and histidine on excretion of (Shapiro and Zwarenstein) 1880
Creatinine, urinary, after castration (Schrire and Zwarenstein) 118
Creatinine, urinary, effect of injection of suspensions of testes and testicular extracts on, after castration (Schrire and Zwarenstein) 1886
Cryptocercus punctulatus, cellulase from intestinal flagellates of (Trager) 1762
Cryptorchidism, effect of, on the gaseous and nitrogenous metabolism of rats (Korenchevsky and Dennison) 429
CUNNINGHAM, R. N. Studies on the chemical nature of secretin 1081
Cysteine, colorimetric determination of (Lugg) 2144, 2160
Cysteine, iodimetric determination of (Lucas and King) 2076
Cystine, colorimetric determination of (Lugg) 2144, 2160
l-Cystine, metabolism of, in the rat (Lawrie) 435

DAJI, J. A. The determination of cellulose in soil 1275
DAMODARAN, M. The isolation of asparagine from an enzymic digest of edestin 235
DAMODARAN, M., JAABACK, G. and CHIBNALL, A. C. The isolation of glutamine from an enzymic digest of gliadin 1704
DANIELLI, J. F., *see* ADAM, N. K.
DANN, W. J. The vitamin D content of red palm oil 151
DANN, W. J. The oxidation of vitamin A *in vitro*. Influence of the solvent 666
DANN, W. J. The transmission of vitamin A from parents to young in mammals 1072
DAOUD, K. M. The reserve polysaccharide of the seeds of fenugreek: its digestibility and its fate during germination 255
DAUPHINEE, J. A., *see* ALLOTT, E. N.
DAVIES, D. R. and DAVIES, W. C. The colorimetric determination of phosphorus in the presence of interfering substances 2046
DAVIES, D. R. and QUASTEL, J. H. Dehydrogenations by brain tissue. The effects of narcotics 1672
DAVIES, D. R., *see* LOCKWOOD, M. R.
DAVIES, W. C., *see* DAVIES, D. R.
DAWSON, E. R. The selective fermentation of glucose and fructose by yeast 531
Dehydrogenase, glucose, product of oxidation of glucose by (Harrison) 1295
Dehydrogenase, triose (Clift and Cook) 1804
Dehydrogenations by brain tissue, effects of narcotics on (Davies and Quastel) 1672
Denaturation of globin (Meldrum) 162
Denaturation of proteins (Loughlin and Lewis) 476
DENNISON, M., *see* KORENCHEVSKY, V.

Dialuric acid, autoxidation of (Richardson) 1959
Dickens, F. and Greville, G. D. The metabolism of normal and tumour tissue. VI. The conversion of fructose and glucose to lactic acid by embryonic tissues 1251
Dickens, F. and Greville, G. D. The metabolism of normal and tumour tissue. VII. The anaerobic conversion of fructose into lactic acid by tumour and adult normal tissues 1546
Dickens, F. and Šimer, F. Metabolism of normal and tumour tissue. V. A note on the carbon dioxide retention of serum: its use in the measurement of tissue glycolysis 90
Diet, stock, for rats bred for vitamin tests (Coward, Cambden and Lee) 679
Diethyldithiocarbamate, sodium, use of, in determination of copper in organic substances (McFarlane) 1022
m-Digallic acid, biological synthesis of (Nierenstein) 1093
Dihydroxybenzenedicarboxylic acids (Birkinshaw and Raistrick) 441
3:5-Dihydroxyphthalic acid, formed from glucose by *P. brevi-compactum* and related species (Oxford and Raistrick) 1902
2:4-Dinitrophenylhydrazones of ketonic acids (Clift and Cook) 1800
Diphtheria toxin and antitoxin, effect of contact with certain surfaces on solutions of (Moloney and Taylor) 1754
Disulphides, colorimetric determination of (Lugg) 2160
Dixon, K. C. and Harrison, K. Glucosone 1954
Dobrowolska, Z. Studies on the catalytic oxidation of uric acid 543
Dodds, E. C. and Gallimore, E. J. The determination of small quantities of oxalic acid 1242
Drummond, J. C., *see* Gillam, A. E. *and* Heilbron, I. M.
Dunlop, D. M., *see* Lyon, D. M.
Dyer, F. J., *see* Coward, K. H.
Dyestuffs, action of, on urease (Quastel) 1685

Eagles, B. A., *see* Sadler, W.
Easson, L. H., *see* Stedman, E.
Eastland, C. J., Evers, N. and Thompson, J. H. Some extracts of parathyroid glands containing an antigrowth factor. I 2123
Eastland, C. J., *see also* Barker, J. H.
Edestin, isolation of asparagine from an enzymic digest of (Damodaran) 235
Edisbury, J. R., Gillam, A. E., Heilbron, I. M. and Morton, R. A. Absorption spectra of substances derived from vitamin A 1164
Egg-albumin, crystalline, basic amino-acids of (Vickery and Shore) 1101
Egg-albumin, effect of addition of electrolytes on viscosity of solutions of (Loughlin) 1557
Elliott, K. A. C. Milk peroxidase. Its preparation, properties and action with H_2O_2 on metabolites. With a method for determining small amounts of H_2O_2 in complex mixtures 10
Elliott, K. A. C. Oxidations catalysed by horseradish- and milk-peroxidases 1281
Ellis, G. W. Autoxidation of the fatty acids. I. The oxygen uptake of elaidic, oleic and stearic acids 791
Ellis, R. W. B., *see* Goldblatt, M. W.
Embryonic tissues, conversion of fructose and glucose into lactic acid by (Dickens and Greville) 1251
Ender, F. The reaction of fish-liver oils with antimony trichloride. (Preliminary communication) 1118
Enterokinase, formation of, from a precursor in the pancreas (Pace) 640, 1566
Enzyme reactions, structure and (Baumgarten) 539
Enzymes, bacterial, liberating molecular hydrogen (Stephenson and Stickland) 712
Enzymic digest of edestin, isolation of asparagine from (Damodaran) 235
Equol (Marrian and Haslewood) 1227
Ergothioneine, determination of, in blood-filtrates (Behre) 458
Ergothioneine, isolation of, from blood (Williamson and Meldrum) 815
Erythrocyte membrane, protein constituent of (Jorpes) 1488
Erythrocytes, mammalian, reduction of glutathione in (Meldrum) 817
Erythrocytes, permeability of (Woodhouse and Pickworth) 309
Essential oils and heat absorption (Nicol) 658
Esterases, activity of, inhibitory action of urethanes on (Stedman and Stedman) 1214
Evans, H. The pentosan content of *Kleinia articulata* 1095
Evers, N., *see* Barker, J. H. *and* Eastland, C. J.

Fat, effect of pituitary on distribution of (Hynd and Rotter) 578
Fat metabolism in fishes (Lovern) 1978, 1985
Fat metabolism of the herring (Channon and Saby) 2021
Fat of fish, fatty acid composition of (Lovern) 1978
Fat, peritoneal, pancreatic and liver, of the sturgeon (Lovern) 1985
Fats, animal depôt, formation and composition of (Banks and Hilditch) 298
Fats, determination of melting-point of (Abbassi) 1857
Fatty acids, autoxidation of (Ellis) 791
Fenugreek seeds, reserve polysaccharide of (Daoud) 255
Fermentation, selective, of glucose and fructose by yeast (Dawson) 531
Fibre-bundles, swelling of single collagen (Marriott) 46
Fine, J. The iodimetric method of determining lactose in milk 569
Fish-liver oils, unsaponifiable fraction of. II (Weidemann) 264
Fish meals, value of, for growth and calcification (Kernot and Speer) 1435
Fixsen, M. A. B. and Jackson, H. M. The biological values of proteins. III. A further note on the method used to measure the nitrogenous excretion of rats 1919
Fixsen, M. A. B. and Jackson, H. M. The biological values of proteins. IV. The biological values of the proteins of wheat, maize and milk 1923

FLEMING, R. The glutathione content of anti-anaemic substances used in the treatment of pernicious anaemia 461
FOLLEY, S. J. The nature of plastein 99
Fomes fomentarius, action of, on wood (Campbell) 1829
FOWWEATHER, F. S. Bilirubin and the van den Bergh reaction 165
FRASER, A. H. H. The reciprocal relationship of calcium and inorganic phosphorus of the blood of sheep 2166
FRED, E. B., *see* TATUM, E. L.
Fructose, anaerobic conversion of, into lactic acid by normal and tumour tissue (Dickens and Greville) 1546
Fructose and glucose, selective fermentation of, by yeast (Dawson) 531
Fructose, conversion of, into lactic acid by embryonic tissue (Dickens and Greville) 1251
FRY, E. G., *see* WOODWARD, G. E.
Fumaric acid, effect of, on deposition of liver-glycogen (Ponsford and Smedley-MacLean) 1340
Fungi, mould, formation of organic acids by (Suthers and Walker) 317
FUNK, C. and ZEFIROW, P. The preparation of gonadotropic hormones from normal urine and urine of pregnancy 619

Gadus aeglefinus, *see* Haddock
GAFFIKIN, M. M., *see* HUME, E. M.
GALLIMORE, E. J., *see* DODDS, E. C.
Ganglion, trigeminal, metabolism of (Holmes) 2005
Ganoderma applanatum, action of, on wood (Campbell) 1829
Gaseous and nitrogenous metabolism of rats, effect of cryptorchidism on (Korenchevsky and Dennison) 429
GAVIN, G., *see* MCHENRY, E. W.
Gelatinase, bacterial, influence of the medium on the production of (Haines) 323
GILLAM, A. E., HEILBRON, I. M., MORTON, R. A. and DRUMMOND, J. C. The isomerisation of carotene by means of antimony trichloride 1174
GILLAM, A. E., *see also* EDISBURY, J. R.
GIRŠAVIČIUS, J. O. Some properties of the enzyme glyoxalase 155
Gliadin, isolation of glutamine from an enzymic digest of (Damodaran, Jaaback and Chibnall) 1704
Globin, denatured (Meldrum) 162
Globin, osmotic pressure of (Roche, Roche, Adair and Adair) 1811
Glucose, absence of abnormal forms of, from blood-dialysates (Bell) 349
Glucose and fructose, selective fermentation of, by yeast (Dawson) 531
Glucose breakdown by pneumococcus variants (Hewitt) 464
Glucose, conversion of, into lactic acid by embryonic tissue (Dickens and Greville) 1251
Glucose, determination of, in broth and in aqueous and peptone solutions (Smith) 1459
Glucose fermentation, relation of thiol compounds to (Quastel and Wheatley) 2169
Glucosone (Dixon and Harrison) 1954
Glutamine, estimation of, in presence of asparagine (Chibnall and Westall) 122
Glutamine, isolation of, from an enzymic digest of gliadin (Damodaran, Jaaback and Chibnall) 1704
Glutathione content of anti-anaemic substances (Fleming) 461
Glutathione, crystallography of (Pirie and Bernal) 75
Glutathione, cuprous (Pirie and Bernal) 75
Glutathione, influence of, on reduction and rotation of blood-filtrates (Groen and Meyer) 624
Glutathione, reduction of, by a liver system (Mann) 785
Glutathione, reduction of, in mammalian erythrocytes (Meldrum) 817
Glyceride fatty acids of forage grasses (Smith and Chibnall) 218
Glycerides of depôt fats of the pig (Banks and Hilditch) 298
Glycine, capillary behaviour of (Jones and Lewis) 638
Glycogen, effect of pituitary on distribution of (Hynd and Rotter) 578
Glycogen, liver, effect of succinic, fumaric, malic and acetic acids on deposition of (Ponsford and Smedley-MacLean) 1340
Glycolysis, tissue, use of carbon dioxide retention of serum in measurement of (Dickens and Simer) 90
Glycosuria, afternoon (Harding, Selby and Armstrong) 957
Glyoxalase, properties of (Giršavičius) 155
GODDEN, W. and ALLCROFT, W. M. Changes in the composition of cow's blood at the time of calving and a comparison of the blood of the calf with that of its dam 1640
GOLDBERG, A., *see* SOBOTKA, H.
GOLDBLATT, M. W. and ELLIS, R. W. B. The metabolism of carbohydrate after starvation 991
Gonadotropic hormones, preparation of, from normal urine and urine of pregnancy (Funk and Zefirow) 619
Gonads, influence of, on protein metabolism (Schrire and Zwarenstein) 118
GOUGH, G. A. C. The specific carbohydrate of the tubercle bacillus 248
GOUGH, G. A. C. A polysaccharide from the pollen of Timothy grass (*Phleum pratense*) 1291
GRAETZ, D., *see* KORENCHEVSKY, V.
GRANT, R. L., SMITH, S. and ZILVA, S. S. Narcotine as the alleged precursor of vitamin C. 1628
Grass, cocksfoot, phosphatides of (Smith and Chibnall) 1345
Grasses, forage, glyceride fatty acids of (Smith and Chibnall) 218
Grasses, proteins of (Miller and Chibnall) 392
GREENE, C. H., WAKEFIELD, E. G., POWER, M. H. and KEITH, N. M. The electrolyte distribution and the acid-base equilibrium in the serum in cases of nephritis and nephritic acidosis 1377
GREENWALD, I. Metabolism in tetany 954
GREVILLE, G. D., *see* DICKENS, F.

GRIFFITHS, W. J. The isolation from bile of a pigment having a direct van den Bergh reaction 1155
GROEN, J. and MEYER, J. W. The influence of glutathione on reduction and rotation of blood-filtrates 624
GROSS, C., *see* SMITH, M. L.
Growth, dietary principle stimulating (Mapson) 970
Growth response of rats, variation of, in vitamin A tests (Coward) 691
Guanidine and derivatives, influence of, on action of amylase (Mystkowski) 910
Guanidine, method for isolation of (Saunders) 801
GULLAND, J. M. The spermicidal activity of quinones and quinols 32
GULLAND, J. M. and NEWTON, W. H. The oxytocic principle of the posterior lobe of the pituitary gland. I 337

HAAS, P. and HILL, T. G. The occurrence of sugar alcohols in marine algae. II. Sorbitol 987
Haddock muscle, after death, carbohydrates and lactic acid of (Macpherson) 80
Haemoglobin, micro-determination of, by blood-benzidine reaction (McFarlane and Hamilton) 1050
HAINES, R. B. The influence of the medium on the production of bacterial gelatinase 323
HAMILTON, R. C. M., *see* MCFARLANE, W. D.
HANES, C. S. Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley 1406
HARDING, V. J., SELBY, D. L., and ARMSTRONG, A. R. Afternoon glycosuria 957
HARRIS, L. J. and RAY, S. N. Vitamin C and the suprarenal cortex. I. Antiscorbutic activity of ox suprarenal 2067
HARRISON, D. C. Note on an accelerating action of uric acid on the xanthine oxidase system 472
HARRISON, D. C. The product of the oxidation of glucose by glucose dehydrogenase 1295
HARRISON, K. Note on the use of sinalbin as an indicator 88
HARRISON, K., *see also* DIXON, K. C.
HASLEWOOD, G. A. D., *see* ADAM, N. K. *and* MARRIAN, G. F.
HASSAN, A., and AYYADI, M. A. S. EL. The effect of hydrolysis on the sugars of normal urine 1851
HASSAN, A. and BASHA, M. K. A. A preliminary note on a prolamin isolated from a legume seed 1843
HASSAN, A. and BASILI, R. The antiscorbutic value of fresh lime juice 1846
HEALEY, M. Note on the supposed production of maclurin from acacatechin 388
Heat absorption, essential oils and (Nicol) 658
HEILBRON, I. M., HESLOP, R. N., MORTON, R. A., WEBSTER, E. T., REA, J. L. and DRUMMOND, J. C. Characteristics of highly active vitamin A preparations 1178
HEILBRON, I. M., MORTON, R. A. and WEBSTER, E. T. The structure of vitamin A 1194
HEILBRON, I. M., *see also* EDISBURY, J. R. *and* GILLAM, A. E.
Hemicelluloses, residual, of rotted straw (Norman) 573
Herring, fat metabolism of (Channon and Saby) 2021
HESLOP, R. N., *see* HEILBRON, I. M.
HEWITT, L. F. Bacterial metabolism. I. Lactic acid production by haemolytic streptococci 208
HEWITT, L. F. Bacterial metabolism. II. Glucose breakdown by pneumococcus variants and the effect of phosphate thereon 464
Hexosediphosphate, sodium, action of *Aspergillus niger* on (Suthers and Walker) 317
Hexosemonophosphate content of rat muscle, effect of adrenaline injections on (Bell) 1601
Hexosemonophosphoric esters (Robison) 2191
HILDITCH, T. P., *see* BANKS, A.
HILL, T. G., *see* HAAS, P.
Histaminase, reaction of, with histamine; purification of, and species distribution of (McHenry and Gavin) 1365
Histidine, colorimetric determination of (Jorpes) 1507
Histidine, effect of administration of, on the excretion of creatinine (Shapiro and Zwarenstein) 1880
HOLMES, E. G. The metabolic activity of the trigeminal ganglion 2005
HOLMES, E. G. Observations on the p_H of brain tissue 2010
HOLMES, E. G. and SHERIF, M. A. F. The relationship between sugar in blood and lactic acid in brain 381
HOPKINS, S. J. and CHIBNALL, A. C. Growth of *Aspergillus versicolor* on higher paraffins 133
Hormones, gonadotropic, preparation of, from normal urine and urine of pregnancy (Funk and Zefirow) 619
Hormones, gonadotropic, purification and properties of (Marshall) 1358
Hormone, testicular, assay of preparations of (Korenchevsky) 413
Hormone, testicular, castrated rats for the assay of (Korenchevsky) 1300
Hormone, testicular, rat unit of (Korenchevsky, Dennison and Kohn-Speyer) 2097
Hormone, testicular, response of castrated male rats to the injection of (Korenchevsky, Dennison and Schalit) 1306
Hormone, yield of testicular, influence of freshness of testes and of desiccation of testicular tissue on (Korenchevsky, Schalit and Graetz) 423
HOUTZ, R. C., *see* NORMAN, A. G.
HUME, E. M., PICKERSGILL, M. and GAFFIKIN, M. M. The determination of vitamin D. I. The relationship between graded doses of a standard solution of vitamin D, administered to young rats on a rachitogenic diet, and the ash content of their bones 488
HURST, R. H. p_H of blood of psychotics measured by the glass electrode 1536
HURTLEY, W. H., *see* ALLOTT, E. N.
Hydrogen ion concentration of blood of psychotics, measured by the glass electrode (Hurst) 1536

Hydrogen ion concentration of brain tissue (Holmes) 2010
Hydrogenlyases (Stephenson and Stickland) 712
Hydrogenlyases, factors concerned in the production of (Yudkin) 1859
Hydrogen peroxide, action of, on metabolites with milk peroxidase (Elliott) 10
Hydrogen peroxide, determination of small amounts of (Elliott) 10
HYND, A. and ROTTER, D. L. Studies on the metabolism of animals on a carbohydrate-free diet. IV. The effect of pitressin and pitocin on the distribution of fat and glycogen in the liver and muscles of albino rats 578
HYND, A. and ROTTER, D. L. Further observations on the distribution of fat and glycogen after the subcutaneous injection of extracts of the posterior pituitary gland 1633
Hyperglycaemia of cancer (Woodward and Fry) 889

Immunological properties of alkali-treated proteins (Johnson and Wormall) 1202
Indan-1 : 3-dione, antiseptics related to (Robinson, Suthers and Walker) 1890
Indicator, use of sinalbin as an (Harrison) 88
INGHAM, J. An improved and simplified benzidine test for blood in urine and other clinical material 1124
Insects, proportion of skeletal tissue in (Buxton) 829
Iodimetric determination of iron in blood (Blackwood and Stirling) 353
Iodimetric determination of lactose in milk (Fine) 569
Iodine, determination of small quantities of, in blood (Allott, Dauphinee and Hurtley) 1665
Iron, determination of, in organic substances (McFarlane) 1034
Iron in blood, iodimetric determination of (Blackwood and Stirling) 353
Iron in relation to tyrosinase (Pugh) 106
Isoelectric point of proteins, effect of addition of salts on (Andrzejewski) 1397

JAABACK, G., *see* DAMODARAN, M.
JACKSON, H. M., *see* CHICK, H. *and* FIXSEN, M. A. B.
JOHNSON, L. R. and WORMALL, A. The immunological properties of alkali-treated proteins 1202
JOHNSON, S. W. and ZILVA, S. S. The antiscorbutic fraction of lemon juice. X 871
JONES, E. B., *see* BARRETT, J. F.
JONES, R. and LEWIS, W. C. M. The physicochemical behaviour of lecithin. II. The capillary activity of lecithin-cholesterol dispersions 633
JONES, R. and LEWIS, W. C. M. Note on the capillary behaviour of glycine 638
JORPES, E. The protein component of the erythrocyte membrane or stroma 1488
JORPES, E. The colorimetric determination of histidine 1507
JORPES, E. and THORÉN, S. The use of the Sakaguchi reaction for the quantitative determination of arginine 1504
JOWETT, M. The reactions of lead compounds with serum and serum-models 2108

KAHN, J., *see* SOBOTKA, H.
Kaufmann-Magne technique, the (Blackwood and Stirling) 357
KEITH, N. M., *see* GREENE, C. H.
KERNOT, J. C. and SPEER, N. E. The relative values of the meals from oily and white fish for growth and calcification 1435
Ketodihydroxyoestrin, conversion of trihydroxyoestrin into (Marrian and Haslewood) 25
Ketones, determination of (Clift and Cook) 1788
Ketonic acids, dinitrophenylhydrazones of (Clift and Cook) 1800
KEY, K. M. and MORGAN, B. G. E. The determination of vitamin D 196
KEY, K. M., *see also* COWARD, K. H.
Kidney, ox, uricase of (Truszkowski) 285
KING, E. J. The colorimetric determination of phosphorus 292
KING, E. J. A titrimetric method for the microdetermination of barium 586
KING, E. J. The action of bone phosphatase on the esters of phosphoric acid of the blood 1697
KING, E. J., *see also* LUCAS, C. C.
KIRK, J. S., *see* SUMNER, J. B.
Kleinia articulata, pentosan content of (Evans) 1095
KODERA, S., *see* SINODA, O.
KOHN-SPEYER, A., *see* KORENCHEVSKY, V.
KORENCHEVSKY, V. The assay of testicular hormone preparations 413
KORENCHEVSKY, V. Castrated rats for the assay of testicular hormone 1300
KORENCHEVSKY, V. and DENNISON, M. A technique for tudying the metabolism of rats 147
KORENCHEVSKY, V. and DENNISON, M. The influence of cryptorchidism on the gaseous and nitrogenous metabolism of rats 429
KORENCHEVSKY, V., DENNISON, M. and KOHN-SPEYER, A. The rat unit of testicular hormone 2097
KORENCHEVSKY, V., DENNISON, M. and SCHALIT, R. The response of castrated male rats to the injection of testicular hormone 1306
KORENCHEVSKY, V., SCHALIT, R. and GRAETZ, D. The influence of the freshness of the testes and of desiccation of the testicular tissue on the yield of testicular hormone 423
KORENCHEVSKY, V., *see also* SAMPSON, M. M.

Lactation, dietary principle stimulating (Mapson) 970
Lactation, mineral metabolism during (Toverud and Toverud) 1424
Lactic acid, anaerobic conversion of fructose into, by normal and tumour tissue (Dickens and Greville) 1546
Lactic acid and carbohydrates of haddock muscle after death (Macpherson) 80
Lactic acid bacteria, nitrogen requirement of (Sadler, Eagles and Pendray) 1532
Lactic acid in brain, relation of, to sugars in blood (Holmes and Sherif) 381
Lactic acid production by certain bacteria, effect of associated growth on (Tatum, Peterson and Fred) 846

Lactic acid production by haemolytic streptococci (Hewitt) 208
Lactic acid production in muscle, determination of (Mawson and Ritchie) 615
Lactose, iodimetric determination of, in milk (Fine) 569
Lactose secretion, relation of blood-sugar absorption to (Blackwood and Stirling) 362
Lange, A. C., *see* West, E. S.
Lawrie, N. R., The metabolism of *i*-cystine in the rat 435
Lawrie, N. R. and Pirie, N. W. Studies in the sulphur metabolism of the dog. X. The effect of *iso*barbituric acid on the excretion of neutral sulphur and ethereal sulphate 622
Lead compounds, reaction of, with serum and serum-models (Jowett) 2108
Leaves of the Brussels sprout, wax metabolism in the (Sahai and Chibnall) 403
Lecithin-cholesterol dispersions, capillary activity of (Jones and Lewis) 633
Lecitho-vitellin, alkaline hydrolysis of (McFarlane) 1061
Lee, E. M., *see* Coward, K. M.
Lemon juice, antiscorbutic fraction of (Johnson and Zilva) 871
Lemon juice, independence of reducing power and antiscorbutic value of (Zilva) 1624
Lewis, W. C. M., *see* Jones, R.
Lime juice, fresh, antiscorbutic value of (Hassan and Basili) 1846
Lipoids, physico-chemical studies of, in relation to salts, drugs and proteins (Spiegel-Adolf) 2183
Liver, effect of pituitary on distribution of fat and glycogen in (Hynd and Rotter) 578
Liver, sheep, effects of freezing and storage on vitamin C contents of (Mills) 704
Liver system, reduction of glutathione by (Mann) 785
Lockwood, M. R. and Davies, D. R. Blood-urea changes in abnormal mental states after the administration of amino-acid 745
Loughlin, W. J. The denaturation of proteins. IX. The effect of the addition of electrolytes on the viscosity of solutions of crystalline egg-albumin 1557
Loughlin, W. J. and Lewis, W. C. M. The denaturation of proteins. VIII. The effect of denaturation on the viscosity of the solutions of certain proteins 476
Lovell, R., *see* Clutterbuck, P. W.
Lovern, J. A. Fat metabolism in fishes. I. General survey of the fatty acid composition of the fats of a number of fishes, both marine and fresh-water 1978
Lovern, J. A. Fat metabolism in fishes. II. The peritoneal, pancreatic and liver fats of the sturgeon (*Acipenser sturio*) 1985
Lucas, C. C. and King, E. J. The iodimetric titration of cysteine and allied substances 2076
Lugg, J. W. H. The application of phospho-18-tungstic acid (Folin's reagent) to the colorimetric determination of cysteine, cystine and related substances. I. The reduction of phospho-18-tungstic acid by various substances 2144
Lugg, J. W. H. The application of phospho-18-tungstic acid (Folin's reagent) to the colorimetric determination of cysteine, cystine and related substances. II. The determination of sulphydryl compounds and disulphides already existing in solution 2160
Lyon, D. M., Dunlop, D. M. and Stewart, C. P. Respiratory quotient in obese subjects 1107

McCance, R. A., *see* Watchorn, E.
McFarlane, W. D. Application of the sodium diethyldithiocarbamate reaction to the micro-colorimetric determination of copper in organic substances 1022
McFarlane, W. D. The determination of iron in organic substances. I. Micro-colorimetry as ferric thiocyanate. II. Potentiometric titration with titanous sulphate 1034
McFarlane, W. D. The alkaline hydrolysis of lecitho-vitellin with particular reference to the separation of an organic fraction containing iron and copper 1061
McFarlane, W. D. and Hamilton, R. C. M. On the factors influencing the blood-benzidine reaction as applied to the micro-determination of haemoglobin 1050
McGowan, J. P. On the fundamental nature of parathormone action 1138
McHenry, E. W. and Gavin, G. Histaminase. I. The histamine-histaminase reaction. II. Purification. III. Species distribution 1365
Mackenzie, K. The biochemistry of aluminium. III. Effect of aluminium on growth and reproduction in the rat. IV. The occurrence of aluminium in the thyroid. V. Intestinal absorption of aluminium in the rabbit 833
Maclurin, supposed production of, from acacatechin (Healey) 388
Macpherson, N. L. Studies in the behaviour of the carbohydrates and lactic acid of the muscle of the haddock (*Gadus aeglefinus*) after death 80
Magnesium and calcium, ultrafiltration of, from human sera (Watchorn and McCance) 54
Maize, biological value of proteins of (Fixsen and Jackson) 1923
Malic acid, effect of, on deposition of liver-glycogen (Ponsford and Smedley-MacLean) 1340
Maltose, determination of, in broth and in aqueous and peptone solutions (Smith) 1459
Mammary gland, absorption of milk precursors by (Blackwood and Stirling) 357, 362; (Blackwood) 772; (Blackwood and Stirling) 778; (Blackwood and Stirling) 1127
Mammary gland, phosphorus metabolism of (Blackwood and Stirling) 778
Mammary gland, relationship of amino-acid absorption to protein synthesis in the (Blackwood) 772
Mann, P. J. G. The reduction of glutathione by a liver system 785
Mannosemonophosphate (Robison) 2191
Mapson, L. W. Evidence of the existence of a dietary principle stimulating general growth and lactation 970

MARRIAN, G. F. and HASLEWOOD, G. A. D. The chemistry of oestrin. V. The mechanism of the conversion of trihydroxyoestrin into ketohydroxyoestrin 25
MARRIAN, G. F. and HASLEWOOD, G. A. D. Equol, a new inactive phenol isolated from the ketohydroxyoestrin fraction of mares' urine 1227
MARRIAN, G. F., *see also* ADAM, N. K.
MARRIOTT, R. H. The swelling of single collagen fibre-bundles 46
MARSHALL, P. G. The gonadotropic hormones (ρ-factors). III. Further purification and properties of the active principles 1358
MATHUR, S. N., *see* ARMITAGE, E.
MAWSON, C. A. and RITCHIE, A. D. A method for the determination of lactic acid production without destruction of the muscle 615
MEDES, G. A new error of tyrosine metabolism: tyrosinosis. The intermediary metabolism of tryosine and phenylalanine 917
MEIKLEJOHN, A. P., PASSMORE, R. and PETERS, R. A. Pyruvic acid and vitamin B_1 deficiency 1872
MELDRUM, N. U. A note on denatured globin 162
MELDRUM, N. U. The reduction of glutathione in mammalian erythrocytes 817
MELDRUM, N. U., *see also* WILLIAMSON, S. W.
Melting-point of fats and allied substances, determination of (Abbassi) 1857
Metabolic products of moulds of the *P. brevi-compactum* series (Clutterbuck, Oxford, Raistrick and Smith) 1441
Metabolism, bacterial (Hewitt) 208, 464
Metabolism, carbohydrate, in human trypanosomiasis (Wormall) 1777
Metabolism, fat, in fishes (Lovern) 1978, 1985
Metabolism, fat, of the herring (Channon and Saby) 2021
Metabolism, gaseous and nitrogenous, of rats, effect of cryptorchidism on (Korenchevsky and Dennison) 429
Metabolism in tetany (Greenwald) 954; (Morris) 1606
Metabolism, intermediary, of tyrosine and phenylalanine (Medes) 917
Metabolism, mineral, during pregnancy and lactation (Toverud and Toverud) 1424
Metabolism of animals on a carbohydrate-free diet (Hynd and Rotter) 578
Metabolism of carbohydrate after starvation (Goldblatt and Ellis) 991
Metabolism of *i*-cystine in the rat (Lawrie) 435
Metabolism of methionine and related compounds in the dog (Pirie) 2041
Metabolism of normal and tumour tissue (Dickens and Simer) 90; (Dickens and Greville) 1251, 1546
Metabolism of rats, technique for studying (Korenchevsky and Dennison) 147
Metabolism of the trigeminal ganglion (Holmes) 2005
Metabolism, phosphorus, of the mammary gland (Blackwood and Stirling) 778
Metabolism, protein, influence of gonads on (Schrire and Zwarenstein) 118, 1886
Metabolism, sulphur, of the dog (Lawrie and Pirie) 622
Metabolism, wax, in the leaves of Brussels sprout (Sahai and Chibnall) 403
Methionine, preparation of, from caseinogen (Pirie) 1270
Methionine, metabolism of, in the dog (Pirie) 2041
Methylation of wood cellulose (Bell) 590
Methylglyoxal, determination of (Clift and Cook) 1788
7-Methylindole, use of, in antimony trichloride test for vitamin A (Morton) 1197
MEYER, J. W., *see* GROEN, J.
Milk, biological value of proteins of (Fixsen and Jackson) 1923
Milk, iodimetric determination of lactose in (Fine) 569
Milk peroxidase (Elliott) 10
Milk precursors, absorption of, by the mammary gland (Blackwood and Stirling) 357, 362; (Blackwood) 772; (Blackwood and Stirling) 778; (Blackwood and Stirling) 1127
Milk-proteins, determination of (Pyne) 1006
Milk secretion, physico-chemical aspects of (Blackwood and Stirling) 1127
MILLER, E. J. and CHIBNALL, A. C. The proteins of grasses. I. Preliminary communication 392
MILLS, J. I. The vitamin C content of sheep-liver; with observations on the effects of freezing and storage 704
MOLONEY, P. J. and TAYLOR, E. M. The effect on solutions of diphtheria toxin and diphtheria antitoxin of contact with certain surfaces 1754
MOORE, T. Vitamin A and carotene. IX. Notes on the conversion of carotene to vitamin A in the cow 1
MORGAN, B. G. E., *see* KEY, K. M. *and* COWARD, K. H.
MORGAN, R. S. The interpretation of the colour match in the antimony trichloride test for vitamin A 377
MORGAN, R. S. The determination of vitamin D in the line test by measurement 1144
MORRIS, N., *see* MORRIS, S.
MORRIS, S. Metabolism in tetany 1606
MORRIS, S. and MORRIS, N. Further observations on the volatile chlorine of blood 2015
MORTON, R. A. The use of 7-methylindole in the antimony trichloride test for vitamin A 1197
MORTON, R. A., *see* COWARD, K. H.; EDISBURY, J. R.; GILLAM, A. E. *and* HEILBRON, I. H.
Mould fungi, formation of organic acids by (Suthers and Walker) 317
Mould tissue, resistant cell-wall material of (Norman and Peterson) 1946
Mould tissue, soluble carbohydrate constituents of (Norman, Peterson and Houtz) 1934
Mucilage of seed of white mustard, composition of (Bailey and Norris) 1609
Muscle, determination of lactic acid production in (Mawson and Ritchie) 615
Muscle, haddock, after death, carbohydrates and lactic acid of (Macpherson) 80
Muscle, origin of pyruvic acid in (Case) 759
Muscles, effects of pituitary on distribution of fat and glycogen in (Hynd and Rotter) 578
MUSGRAVE, F. F., *see* YOUNG, E. G.

Mustard, white, composition of mucilage of seed of (Bailey and Norris) 1609
MYSTKOWSKI, E. M. Influence of guanidine and its derivatives on the action of amylase 910

NANAVUTTY, S. H. Van Slyke's method of determination of acetone bodies applied to small volumes of blood and urine 1391
NANJI, H. R., *see* BUSTON, H. W.
Narcotics, effects of, on dehydrogenations by brain tissue (Davies and Quastel) 1672
Narcotine, as alleged precursor of vitamin C (Grant, Smith and Zilva) 1628
Nephritis, electrolyte distribution and acid-base equilibrium in serum in cases of (Greene, Wakefield, Power and Keith) 1377
NEWTON, W. H., *see* GULLAND, J. M.
NICOL, H. Essential oils and heat absorption 658
NIELSEN, E., *see* BIERRING, E.
NIERENSTEIN, M. A biological synthesis of *m*-digallic acid 1093
Nitrogenous and gaseous metabolism of rats, effect of cryptorchidism on (Korenchevsky and Dennison) 429
Nitrogenous exchange of rats, method of measurement of (Fixsen and Jackson) 1919
NORMAN, A. G. The biological decomposition of plant materials. VII. The nature of the residual hemicelluloses of rotted straw 573
NORMAN, A. G., PETERSON, W. H. and HOUTZ, R. C. The chemistry of mould tissue. I. Soluble carbohydrate constituents 1934
NORMAN, A. G. and PETERSON, W. H. The chemistry of mould tissue. II. The resistant cell-wall material 1946
NORRIS, F. W., *see* BAILEY, K.

Obese subjects, respiratory quotient in (Lyon, Dunlop and Siewart) 1107
Obituary notice of A. C. Chapman 1715
Obituary notice of S. J. B. Connell 1718
Obituary notice of W. D. Halliburton 269
Obituary notice of Morna Macleod 271
Obituary notice of M. W. Onslow 915
Obituary notice of M. J. Rowlands 1718
Obituary notice of R. Stenhouse Williams 537
O'BRIEN, J. R. P., *see* BARNES, H.
Oestrin, chemistry of. V. (Marrian and Haslewood) 25
Oestrin derivatives, surface films of (Adam, Danielli, Haslewood and Marrian) 1233
Oils, fish-liver, reaction of, with antimony trichloride (Ender) 1118
Osmotic pressure of globin (Roche, Roche, Adair and Adair) 1811
Oxalic acid, determination of small quantities of (Dodds and Gallimore) 1242
OXFORD, A. E. and RAISTRICK, H. Studies in the biochemistry of micro-organisms. XXV. 3 : 5-Dihydroxyphthalic acid, a new product of the metabolism of glucose by *Penicillium brevi-compactum* and related species 1902
OXFORD, A. E., *see also* CLUTTERBUCK, P. W.
Oxidase system, xanthine, accelerating effect of uric acid on the (Harrison) 472
Oxidation of uric acid, catalytic (Dobrowolska) 543
Oxidation of vitamin A *in vitro*, influence of the solvent on (Dann) 666
Oxidations by the brain (Quastel and Wheatley) 725
Oxygen uptake of brain tissue, effect of alcohol on (Robertson and Stewart) 65
Oxytocic principle of the posterior lobe of the pituitary gland (Gulland and Newton) 337

PACE, J. The formation of enterokinase from a precursor in the pancreas 640
PACE, J. The formation of enterokinase from a precursor in the pancreas: a supplementary note 1566
PAGE, I. H. and YOUNG, F. G. The effect of intravenous phosphatide on the sugar excretion of the phloridzinised dog 1528
Palm oil, red, vitamin D content of (Dann) 151
Pancreas, formation of enterokinase from a precursor in the (Pace) 640
Papain, immunological reaction to (Walton and Segura) 1750
Paraffins, higher, growth of *Aspergillus versicolor* on (Hopkins and Chibnall) 133
Parathormone action, fundamental nature of (McGowan) 1138
Parathyroid glands, antigrowth factor in extracts of (Eastland, Evers and Thompson) 2123
PASSMORE, R., *see* MEIKLEJOHN, A. P.
PATERSON, A. R. C., *see* BELL, G. H.
Pectic acid, preparation of methyl esters of (Buston and Nanji) 2090
PENDRAY, G., *see* SADLER, W.
PENFOLD, W. J. and SUTHERLAND, J. A note on movements and precipitations of an amphoteric colloid due to an electric field and slight electrolysis. A modified Hardy's experiment suitable for demonstration purposes 1839
Penicillin, produced from glucose by *P. chrysogenum* (Clutterbuck, Lovell and Raistrick) 1907
Penicillium brevi-compactum, formation of 3:5-dihydroxyphthalic acid from glucose by (Oxford and Raistrick) 1902
Penicillium brevi-compactum series, metabolic products of (Clutterbuck, Oxford, Raistrick and Smith) 1441
Penicillium chrysogenum, products of action of, on glucose (Clutterbuck, Lovell and Raistrick) 1907
Penicillium, species of, production of puberulic acid and an acid $C_8H_4O_6$ by, from glucose (Birkinshaw and Raistrick) 441
PENTELOW, F. T. K., *see* SOUTHGATE, B. A.
Pentosan content of *Kleinia articulata* (Evans 1095
Pepsin, hydrolysis of caseinogen by (Stirling and Wishart) 1989
Permeability of vital membranes. The red blood corpuscle (Woodhouse and Pickworth) 309
Peroxidase, milk (Elliott) 10, 1281
Peroxidases of horseradish and milk, oxidations catalysed by (Elliott) 1281
PETERS, R. A., *see* BARNES, H. *and* MEIKLEJOHN, A. P.
PETERSON, W. H., *see* NORMAN, A. G. *and* TATUM, E. L.

PETERSON, V. L., *see* WEST, E. S.
p_H chart, a (Bell and Paterson) 454
Phenylalanine, intermediary metabolism of (Medes) 917
Phenylalanine, oxidation of, with hydrogen peroxide (Raper) 2000
Phleum pratense, polysaccharide from the pollen of (Gough) 1291
Phloridzinised dog, effect of intravenous phosphatide on sugar excretion of (Page and Young) 1528
Phosphatase, bone, action of, on phosphoric esters of blood (King) 1697
Phosphate, effect of, on glucose breakdown by pneumococcus variants (Hewitt) 464
Phosphatide, intravenous, effect of, on sugar excretion of phloridzinised dog (Page and Young) 1528
Phosphatides of cocksfoot grass (Smith and Chibnall) 1345
Phosphoric esters of blood, action of bone phosphatase on (King) 1697
Phosphorus, colorimetric determination of (King) 292
Phosphorus, determination of, in presence of interfering substances (Davies and Davies) 2046
Phosphorus, inorganic, and calcium of sheep's blood, reciprocal relationship of (Fraser) 2166
Phosphorus metabolism of the mammary gland (Blackwood and Stirling) 778
Phospho-18-tungstic acid, reduction of (Lugg) 2144
Phospho-18-tungstic acid, use of, in colorimetric determination of cysteine, cystine and related substances (Lugg) 2144, 2160
Phospho-24-tungstic acid, method of checking the purity of (Barnes and Peters) 2203
PICKERING, J. W., *see* ARMITAGE, E.
PICKERSGILL, M., *see* HUME, E. M.
PICKWORTH, F. A., *see* WOODHOUSE, D. L.
Pig, body fats of (Banks and Hilditch) 298
Pigment, produced from glucose by *P. chrysogenum* (Clutterbuck, Lovell and Raistrick) 1907
PIRIE, N. W. The preparation of methionine from caseinogen 1270
PIRIE, N. W. Studies in the sulphur metabolism of the dog. XI. The metabolism of methionine and related sulphides 2041
PIRIE, N. W. and BERNAL, J. D. Cuprous glutathione. With a note on the crystallography of glutathione 75
PIRIE, N. W., *see also* LAWRIE, N. R.
Pituitary gland, anterior, composition of tissues of albino rats treated with alkaline extracts of (Bierring and Nielson) 1015
Pituitary gland, effect of, on distribution of fat and glycogen in liver and muscles of albino rats (Hynd and Rotter) 578
Pituitary gland, oxytocic principle of the posterior lobe of the (Gulland and Newton) 337
Pituitary gland, posterior, distribution of fat and glycogen after injection of extracts of (Hynd and Rotter) 1633
Plant materials, biological decomposition of (Norman) 573
Plastein, the nature of (Folley) 99
Pleurotus ostreatus, action of, on wood (Campbell) 1829
Pneumococcus variants, breakdown of glucose by (Hewitt) 464
Polyporus adustus, action of, on wood (Campbell) 1829
Polysaccharide from the pollen of Timothy grass (Gough) 1291
Polysaccharide, reserve, of fenugreek seeds (Daoud) 255
Polystictus abietinus, action of, on wood (Campbell) 1829
PONSFORD, A. P. and SMEDLEY-MACLEAN, I. The influence of succinic, fumaric, malic and acetic acids on the deposition of liver-glycogen 1340
POWER, M. H., *see* GREENE, C. H.
Pregnancy, mineral metabolism during (Toverud and Toverud) 1424
Pregnancy urine, bromine reaction of (Armstrong and Walker) 143
Prolamin, from a legume seed (Hassan and Basha) 1843
Pro-lipase, pancreatic, activation of (Woodhouse) 1512
Protein, alkali-soluble, produced from glucose by *P. chrysogenum* (Clutterbuck, Lovell and Raistrick) 1907
Protein constituent of erythrocyte membrane (Jorpes) 1488
Protein, determination of basic amino-acids in small amounts of (Rosedale and da Silva) 369
Protein metabolism, influence of gonads on (Schrire and Zwarenstein) 118, 1886
Proteins, alkali-treated, immunological properties of (Johnson and Wormall) 1202
Proteins, isoelectric point of, effect of addition of salts on (Andrzejewski) 1397
Proteins, milk, determination of (Pyne) 1006
Proteins, nitrogen content of certain (Smith, Brown and Gross) 1473
Proteins, of blood, determination of, by acid-acetone (Race) 1571
Proteins of grasses (Miller and Chibnall) 392
Proteins of wheat, maize and milk, biological values of (Fixsen and Jackson) 1923
Proteins, serum, precipitation and protection of vanadic acid by (Boyland) 1383
Proteins, solutions of, effect of denaturation on viscosity of (Loughlin and Lewis) 476
Protein—starch—amylase systems, effect of salts on (Baumgarten) 539
Protein synthesis in the mammary gland, relationship of amino-acid absorption to (Blackwood) 772
Proteolysis in tumours (Rondini) 1477
Psychotics, p_H of blood of (Hurst) 1536
Puberulic acid and an acid $C_8H_4O_6$ from metabolism of glucose by species of *Penicillium* (Birkinshaw and Raistrick) 441
PUGH, C. E. M. Iron in relation to tyrosinase 106
PYNE, G. T. The determination of milk-proteins by formaldehyde titration 1006
Pyruvic acid and vitamin B_1 deficiency (Meiklejohn, Passmore and Peters) 1872
Pyruvic acid, determination of (Case) 753; (Clift and Cook) 1788
Pyruvic acid, origin of, in muscle (Case) 759

QUASTEL, J. H. The action of dyestuffs on enzymes. III. Urease 1685
QUASTEL, J. H. and WHEATLEY, A. H. M. Oxidations by the brain 725
QUASTEL, J. H. and WHEATLEY, A. H. M. The relation of thiol compounds to glucose fermentation 2169
QUASTEL, J. H., *see also* DAVIES, D. R.
Quinones and quinols, spermicidal activity of (Gulland) 32

RABINOWITCH, I. M. The relative proportions of fermentable and non-fermentable reducing substances of hyperglycaemic bloods of diabetics without glycosuria 963
RACE, J. The determination of blood-proteins by acid-acetone 1571
Rachitogenic diet, relation of graded doses of vitamin D to ash content of bones of rats on a (Hume, Pickersgill and Gaffikin) 488
Radiography and bone analysis, comparison of, in the estimation of vitamin D (Bourdillon and Bruce) 506
RAISTRICK, H., *see* BIRKINSHAW, J. H., CLUTTERBUCK, P. W. *and* OXFORD, A. E.
RAPER, H. S. Note on the oxidation of tyrosine, tyramine and phenylalanine with hydrogen peroxide 2000
Rat unit of testicular hormone (Korenchevsky, Dennison and Kohn-Speyer) 2097
RAY, S. N., *see* HARRIS, L. J.
REA, J. L., *see* HEILBRON, I. M.
READER, V., *see* BARNES, H.
Reducing substances of hyperglycaemic bloods of diabetics without glycosuria (Rabinowitch) 963
Respiratory quotient in obese subjects (Lyon, Dunlop and Stewart) 1107
RICHARDSON, G. M. The autoxidation of dialuric acid 1959
RITCHIE, A. D., *see* MAWSON, C. A.
ROBERTSON, J. D. and STEWART, C. P. The effect of alcohol on the oxygen uptake of brain tissue 65
ROBINSON, F. A., SUTHERS, A. J. and WALKER, T. K. Some new antiseptics related to indan-1 : 3-dione 1890
ROBINSON, G. M. and ROBINSON, R. A survey of anthocyanins 1647
ROBINSON, R., *see* ROBINSON, G. M.
ROBISON, R. Hexosemonophosphoric esters: mannosemonophosphate 2191
ROCHE, A., *see* ROCHE, J.
ROCHE, J., ROCHE, A., ADAIR, G. S. and ADAIR, M. E. The osmotic pressure of globin 1811
RONDONI, P. Proteolysis in tumours 1477
ROSEDALE, J. L. and da SILVA, G. A. The amino-acids of tissues. VI. Determination of the basic amino-acids in small quantities of protein 369
ROTTER, D. L., *see* HYND, A.
Ryegrass, perennial, glyceride fatty acids of (Smith and Chibnall) 218

SABY, M. K. EL, *see* CHANNON, H. J.
SADLER, W., EAGLES, B. A. and PENDRAY, G. Cheese-ripening studies. II. The nitrogen requirement of the lactic acid bacteria 1532
SAHAI, P. N. and CHIBNALL, A. C. Wax metabolism in the leaves of Brussels sprout 403
Sakaguchi reaction, determination of arginine by use of (Jorpes and Thorén) 1504
SAMPSON, M. M., DENNISON, M. and KORENCHEVSKY, V. The absorption of nitrogen and of fat from the alimentary canal of rats kept on a vitamin A-deficient diet 1315
SAMPSON, M. M. and KORENCHEVSKY, V. The influence of vitamin A deficiency on male rats in paired feeding experiments 1322
SAMPSON, M. M. and KORENCHEVSKY, V. Vitamin A deficiency in castrated male rats 1542
SAMPSON, M. M., *see also* KORENCHEVSKY, V.
SAUNDERS, J. A. A method for the isolation of guanidine 801
SCHALIT, R., *see* KORENCHEVSKY, V.
SCHRIRE, I. and ZWARENSTEIN, H. The influence of the gonads on protein metabolism. I. Urinary creatinine after castration 118
SCHRIRE, I. and ZWARENSTEIN, H. The influence of the gonads on protein metabolism. II. The effect of injections of saline suspensions of testes and testicular extracts on urinary creatinine after castration 1886
Secretin, chemical nature of (Cunningham) 1081
SEGURA, C. M., *see* WALTON, R. P.
SELBY, D. L., *see* HARDING, V. J.
Serum, human, ultra-filtration of calcium and magnesium from (Watchorn and McCance) 54
Serum-models, reaction of, with lead compounds (Jowett) 2108
Serum, reaction of, with lead compounds (Jowett) 2108
Serum, use of carbon dioxide retention of, in measurement of tissue glycolysis (Dickens and Šimer) 90
SHAPIRO, B. G. and ZWARENSTEIN, H. The influence of the administration of arginine and histidine on the excretion of creatinine 1880
SHERIF, M. A. F., *see* HOLMES, E. G.
SHORE, A., *see* VICKERY, H. B.
DA SILVA, G. A., *see* ROSEDALE, J. L.
ŠIMER, F., *see* DICKENS, F.
Sinalbin as an indicator, use of (Harrison) 88
SINODA, O. and KODERA, S. The chemistry of cooking. II. On the critical temperature in cooking the sweet potato 650
Skeletal tissue, proportion of, in insects (Buxton) 829
SMEDLEY-MACLEAN, I., *see* PONSFORD, A. P.
SMITH, G., *see* CLUTTERBUCK, P. W.
SMITH, J. A. B. and CHIBNALL, A. C. The glyceride fatty acids of forage grasses. I. Cocksfoot and perennial ryegrass 218
SMITH, J. A. B. and CHIBNALL, A. C. The phosphatides of forage grasses. I. Cocksfoot 1345
SMITH, M. L. The determination of glucose and maltose in aqueous solution and in broth and peptone solutions 1459
SMITH, M. L. The effect of heat on sugar solutions used for culture media 1467
SMITH, M. L., BROWN, A. M. and GROSS, C. Note on the nitrogen content of certain proteins 1473
SMITH, S., *see* GRANT, R. L.

Smolts, causes of death of, in Tees estuary (Southgate, Pentelow and Bassindale) 273
SOBOTKA, H. and GOLDBERG, A. Choleic acids 555
SOBOTKA, H. and GOLDBERG, A. Choleic acids. V. The separation of optical antipodes by means of co-ordination compounds 905
SOBOTKA, H. and KAHN, J. Choleic acids. IV. Keto-enol tautomerism and co-ordination compounds 898
Sorbitol, occurrence of, in marine algae (Haas and Hill) 987
SOUTHGATE, B. A., PENTELOW, F. T. K., and BASSINDALE, R. An investigation into the causes of death of salmon and sea trout smolts in the estuary of the river Tees 273
Spectra, absorption, of substances derived from vitamin A (Edisbury, Gillam, Heilbron and Morton) 1164
SPEER, N. E., *see* KERNOT, J. C.
Spermicidal activity of quinones and quinols (Gulland) 32
SPIEGEL-ADOLF, M. Physico-chemical studies of lipoids in their relations to salts, drugs and proteins 2183
Starch—amylase—protein systems, effect of salts on (Baumgarten) 539
Starvation, metabolism of carbohydrate after (Goldblatt and Ellis) 991
STEDMAN, E. and STEDMAN, E. Studies on the relationship of chemical constitution and physiological action. IV. The inhibitory action of certain synthetic urethanes on the activity of esterases 1214
STEDMAN, E., STEDMAN, E. and EASSON, L. H. Choline-esterase. An enzyme present in the blood-serum of the horse 2056
STEINER, A., *see* WEST, E. S.
STEPHENSON, M. and STICKLAND, L. H. Hydrogenlyases. Bacterial enzymes liberating molecular hydrogen . 712
STEWART, C. P., *see* LYON, D. M., *and* ROBERTSON, J. D.
STICKLAND, L. H., *see* STEPHENSON, M.
STIRLING, J. D. and WISHART, G. M. The hydrolysis of caseinogen by pepsin and by trypsin-kinase 1989
STIRLING, J. D., *see also* BLACKWOOD, J. H.
Straw, rotted, residual hemicellulose of (Norman) 537
Streptococci, haemolytic, lactic acid production by (Hewitt) 208
Sturgeon, peritoneal, pancreatic and liver fats of (Lovern) 1985
Succinic acid, effect of, on deposition of liver-glycogen (Ponsford and Smedley-MacLean) 1340
Sulphydryl compounds, colorimetric determination of (Lugg) 2160
Sugar in blood and lactic acid in brain, relation of (Holmes and Sherif) 381
Sugar, fermentable, of normal and starvation urine, chemical nature of (West and Steiner) 1742
Sugar solutions used for culture media, effect of heat on (Smith) 1467
Sugars, fermentable and non-fermentable, factors affecting the excretion of, in urine (West, Lange and Peterson) 1728
Sugars of normal urine, effect of hydrolysis on (Hassan and Ayyadi) 1851
Sugars, reducing, of urine, determination of (West and Peterson) 1720
Sulphur, neutral, and ethereal sulphate, effect of *iso*barbituric acid on excretion of (Lawrie and Pirie) 622
SUMNER, J. B. and KIRK, J. S. Is there a co-enzyme for urease? 551
Suprarenal cortex, vitamin C in (Harris and Ray) 2067
Suprarenal gland of ox, antiscorbutic activity of cortex of (Zilva) 2182
Surface films of oestrin derivatives (Adam, Danielli, Haslewood and Marrian) 1233
SUTHERLAND, J., *see* PENFOLD, W. J.
SUTHERS, A. J. and WALKER, T. K. The mechanism of the formation of organic acids by mould fungi. I. The action of *Aspergillus niger* on sodium hexosediphosphate in the presence of toluene 317
SUTHERS, A. J., *see also* ROBINSON, F. A.
Swelling of single collagen fibre-bundles (Marriott) 46
SVIRBELY, J. L. and SZENT-GYÖRGYI, A. The chemical nature of vitamin C 865
SZENT-GYÖRGYI, A., *see* SVIRBELY, J. L.

TATUM, E. L., PETERSON, W. H. and FRED, E. B. Effect of associated growth on forms of lactic acid produced by certain bacteria 846
TAYLOR, E. M., *see* MOLONEY, P. J.
Tees estuary, causes of death of smolts in (Southgate, Pentelow and Bassindale) 273
Termites, cellulose from intestinal flagellates of (Trager) 1762
Testes, suspension of, and testicular extracts, effect of injection of, on urinary creatinine after castration (Schrire and Zwarenstein) 1886
Testicular hormone preparations, assay of (Korenchevsky) 413
Testicular hormone, the rat unit of (Korenchevsky, Dennison and Kohn-Speyer) 2097
Testicular hormone, yield of, influence of freshness of testes and of desiccation of testicular tissue on (Korenchevsky, Schalit and Graetz) 423
Tetany, metabolism in (Greenwald) 954; (Morris) 1606
Thiol compounds, relation of, to glucose fermentation (Quastel and Wheatley) 2169
THOMPSON, J. H., *see* EASTLAND, C. J.
THORÉN, S., *see* JORPES, E.
Thyroid, occurrence of aluminium in (Mackenzie) 833
Timothy grass, polysaccharide from the pollen of (Gough) 1291
Tissue glycolysis, use of carbon dioxide retention of serum in measurement of (Dickens and Šimer) 90
Tissues, amino-acids of (Rosedale and da Silva) 369
TOVERUD, K. U. and TOVERUD, G. Studies on the mineral metabolism during pregnancy and lactation. II 1424
TOVERUD, G., *see* TOVERUD, K. U.

TRAGER, W. A cellulase from the symbiotic intestinal flagellates of termites and of the roach, *Cryptocercus punctulatus* 1762
Trametes pini, action of, on wood (Campbell) 1829
Trihydroxyoestrin, conversion of, into ketodihydroxyoestrin (Marrian and Haslewood) 25
Triose dehydrogenase (Clift and Cook) 1804
TRUSZKOWSKI, R. Uricase and its action. V. Further examination of ox-kidney uricase 285
Trypanosomiasis, human, carbohydrate metabolism in (Wormall) 1777
Trypsin-kinase, hydrolysis of caseinogen by (Stirling and Wishart) 1989
Tubercle bacillus, specific carbohydrate of (Gough) 248
Tumours, proteolysis in (Rondini) 1477
Tyramine, oxidation of, with hydrogen peroxide (Raper) 2000
Tyrosinase, iron in relation to (Pugh) 106
Tyrosine, intermediary metabolism of (Medes) 917
Tyrosine, oxidation of, with hydrogen peroxide (Raper) 2000
Tyrosinosis (Medes) 917

Urate gels (Young and Musgrave) 941
Urea, colorimetric method for direct determination of, in urine (Barrett and Jones) 1246
Urease, action of dyestuffs on (Quastel) 1685
Urease, question of a co-enzyme for (Sumner and Kirk) 551
Urethanes, inhibitory action of, on activity of esterases (Stedman and Stedman) 1214
Uric acid, accelerating action of, on xanthine oxidase system (Harrison) 472
Uric acid, catalytic oxidation of (Dobrowolska) 543
Uricase, ox-kidney (Truszkowski) 285
Urine, bromine reaction of pregnancy (Armstrong and Walker) 143
Urine, determination of acetone bodies in small volumes of (Nanavutty) 1391
Urine, determination of reducing sugars of (West and Peterson) 1720
Urine, direct colorimetric determination of urea in (Barrett and Jones) 1246
Urine, factors affecting the excretion of fermentable and non-fermentable sugars in (West, Lange and Peterson) 1728
Urine, normal and starvation, chemical nature of fermentable sugar of (West and Steiner) 1742
Urine, normal, effect of hydrolysis on sugars of (Hassan and Ayyadi) 1851
Urine, preparation of gonadotropic hormones from (Funk and Zefirow) 619
van den Bergh reaction, bilirubin and the (Fowweather) 165
van den Bergh reaction, direct, of a pigment from bile (Griffiths) 1155
Vanadic acid, precipitation and protection of, by serum-proteins (Boyland) 1383
VICKERY, H. B. and SHORE, A. The basic amino-acids of crystalline egg-albumin 1101
Viscosity of solutions of egg-albumin, effect on, of the addition of electrolytes (Loughlin) 1557
Viscosity of solutions of proteins, effect of denaturation on (Loughlin and Lewis) 476
Vitamin A, absorption of nitrogen and fat from the alimentary canal of rats on a diet deficient in (Sampson, Dennison and Korenchevsky) 1315
Vitamin A, absorption spectra of substances derived from (Edisbury, Gillam, Heilbron and Morton) 1164
Vitamin A, conversion of carotene into, in the cow (Moore) 1
Vitamin A deficiency, effect of, on male rats in paired feeding experiments (Sampson and Korenchevsky) 1322
Vitamin A deficiency in castrated male rats (Sampson and Korenchevsky) 1542
Vitamin A, determination of, in cod-liver oils by measurements of absorption in the ultraviolet (Coward, Dyer and Morton) 1593
Vitamin A, highly active preparations of, characteristics of (Heilbron, Heslop, Morton, Webster, Rea and Drummond) 1178
Vitamin A, interpretation of the colour match in the antimony trichloride test for (Morgan) 377
Vitamin A, oxidation of, *in vitro* (Dann) 666
Vitamin A, structure of (Heilbron, Morton and Webster) 1194
Vitamin A tests, variation in growth response of rats in (Coward) 691
Vitamin A, transmission of, from parents to young in mammals (Dann) 1072
Vitamin A, use of 7-methylindole in antimony trichloride test for (Morton) 1197
Vitamin B_1 deficiency, pyruvic acid and (Meiklejohn, Passmore and Peters) 1872
Vitamin B_1, International Standard for (Chick and Jackson) 1223
Vitamin B_1 (Barnes, O'Brien and Reader) 2035
Vitamin C, chemical nature of (Svirbely and Szent-Györgyi) 865
Vitamin C content of sheep-liver, effects of freezing and storage on (Mills) 704
Vitamin C, narcotine as alleged precursor of (Grant, Smith and Zilva) 1628
Vitamin C, presence of, in suprarenal cortex (Harris and Ray) 2067
Vitamin C, *see also* Antiscorbutic
Vitamin D, comparison of radiography and bone analysis in estimation of (Bourdillon and Bruce) 506
Vitamin D content of red palm oil (Dann) 151
Vitamin D, determination of (Key and Morgan) 196; (Hume, Pickersgill and Gaffikin) 488; (Bourdillon and Bruce) 506; (Bourdillon, Bruce and Webster) 522; (Coward, Key and Morgan) 1585
Vitamin D, determination of, in the line test by measurement (Morgan) 1144
Vitamin D, production of, in a glow discharge (Askew, Bourdillon and Webster) 814
Vitamin D, relation of graded doses of, to ash content of bones of rats on rachitogenic diet (Hume, Pickersgill and Gaffikin) 488
Vitamin D, stability of preparations of (Bourdillon, Bruce and Webster) 522

Vitamin tests, a stock diet for rats bred for (Coward, Cambden and Lee) 679

WAKEFIELD, E. G., *see* GREENE, C. H.
WALKER, E., *see* ARMSTRONG, A. R.
WALKER, T. K., *see* ROBINSON, F. A. *and* SUTHERS, A. J.
WALTON, R. P. and SEGURA, C. M. The immunological reaction to the enzyme papain 1750
WATCHORN, E. and MCCANCE, R. A. Inorganic constituents of cerebrospinal fluid. II. The ultrafiltration of calcium and magnesium from human sera 54
Wax metabolism in the leaves of the Brussels sprout (Sahai and Chibnall) 403
WEBSTER, E. T., *see* HEILBRON, I. M.
WEBSTER, T. A., *see* ASKEW, F. A. *and* BOURDILLON, R. B.
WEIDEMANN, C. (the late). Studies on the chemical nature of the unsaponifiable fraction of fish-liver oils. II. Cod-liver oil. Edited by F. Ender 264
WEST, E. S., LANGE, A. C. and PETERSON, V. L. The sugars of urine. II. Factors affecting the excretion of fermentable and non-fermentable sugars in urine 1728
WEST, E. S. and PETERSON, V. L. The sugars of urine. I. Determination of the reducing sugars of urine 1720
WEST, E. S. and STEINER, A. The sugars of urine. III. The chemical nature of the fermentable sugar of normal and starvation urine 1742
WESTALL, R. G., *see* CHIBNALL, A. C.
Wheat, biological value of proteins of (Fixsen and Jackson) 1923
WHEATLEY, A. H. M., *see* QUASTEL, J. H.
White rots of wood, chemistry of (Campbell) 1829
WILLIAMSON, S. W. and MELDRUM, N. U. A note on the isolation of ergothioneine from blood 815
WISHART, G. M., *see* STIRLING, J. D.
Wood cellulose (Bell) 590, 598, 609
WOODHOUSE, D. L. Investigations in enzyme action directed towards the study of the biochemistry of cancer. The activation of pancreatic pro-lipase 1512
WOODHOUSE, D. L. and PICKWORTH, F. A. Permeability of vital membranes. The red blood corpuscle 309
WOODWARD, G. E. and FRY, E. G. The hyperglycaemia of cancer 889
WORMALL, A. Carbohydrate metabolism in human trypanosomiasis 1777
WORMALL, A., *see also* JOHNSON, L. R.

Xanthine oxidase system, accelerating effect of uric acid on the (Harrison) 472

Yeast, selective fermentation of glucose and fructose by (Dawson) 531
YOUNG, E. G. and MUSGRAVE, F. F. The formation and decomposition of urate gels 941
YOUNG, F. G., *see also* PAGE, I. H.
YUDKIN, J. Hydrogenlyases. II. Some factors concerned in the production of the enzymes 1859

ZEFIROW, P., *see* FUNK, C.
ZILVA, S. S. The non-specificity of the phenol-indophenol reducing capacity of lemon juice and its fractions as a measure of their antiscorbutic capacity 1624
ZILVA, S. S. The antiscorbutic activity of the cortex of the suprarenal gland of the ox 2182
ZILVA, S. S., *see also* CRANE, M. B., GRANT, R. L. *and* JOHNSON, S. W.
ZWARENSTEIN, H., *see* SCHRIRE, I. *and* SHAPIRO, B. G.

CAMBRIDGE: PRINTED BY WALTER LEWIS, M.A., AT THE UNIVERSITY PRESS

PRICES OF BACK NUMBERS AND VOLUMES.

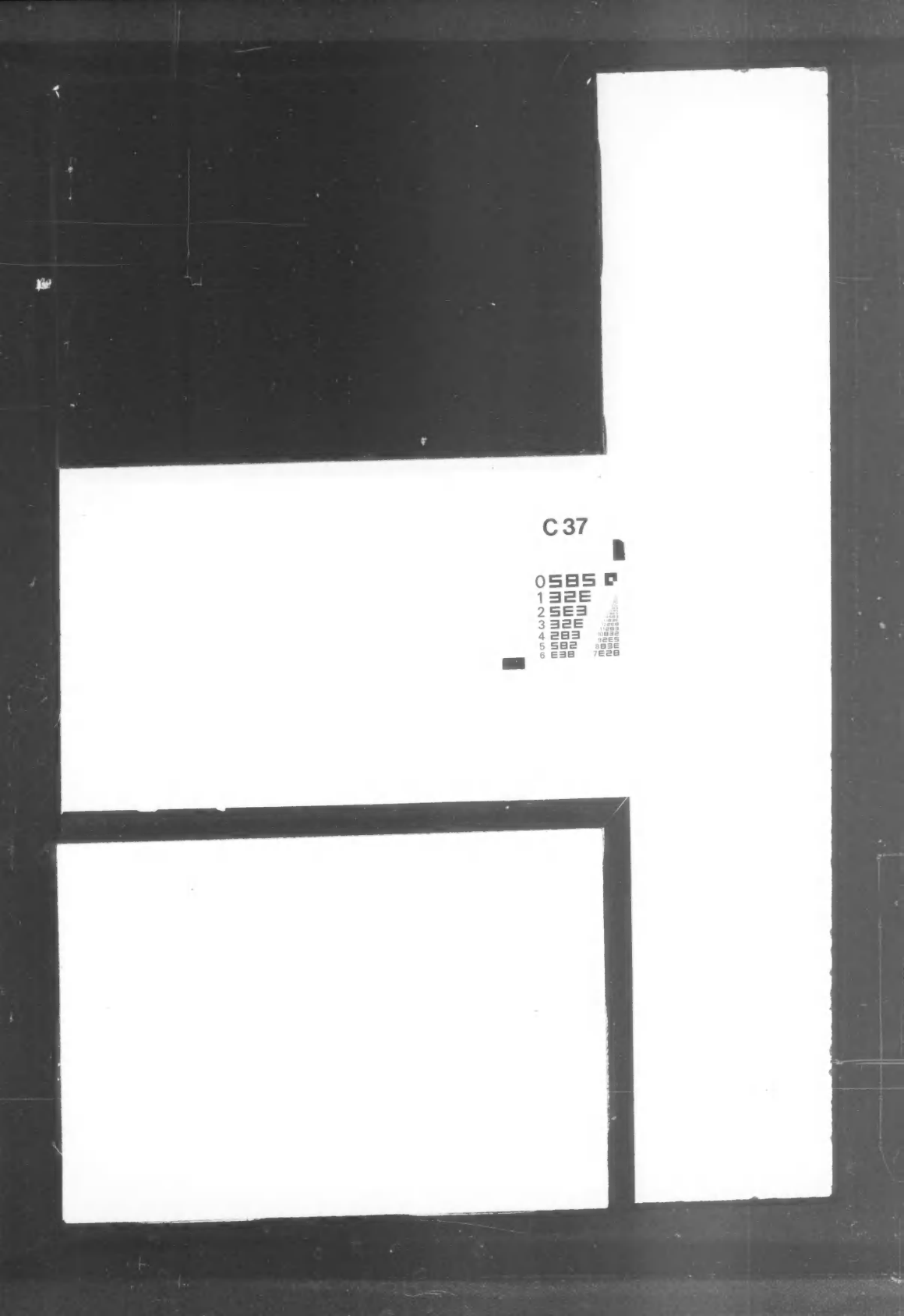
In Paper covers.

Volumes I–VI.	31*s.* 6*d.* net per vol.	Quotations can be given by the publishers for bound copies of back numbers.
Volumes VII–X.	(*Out of print.*)	
Index of Authors and Subjects in Volumes I–X.	5*s.* 3*d.* net. (By post, 5*s.* 5*d.*)	
Volumes XI–XVI.	(*Out of print.*)	
Volumes XVII–XVIII.	60*s.* net per vol.	
Volume XIX.	(*Out of print.*)	
Volumes XX–XXV.	60*s.* net per vol.	
Index of Authors and Subjects in Volumes XI–XX.	10*s.* net. (By post, 10*s.* 4*d.*)	

Claims for Journals lost in Post will not be entertained more than 3 months after the date of posting of the Journal.

For Notice to Contributors see page 2 of Wrapper

PRINTED IN GREAT BRITAIN BY W. LEWIS, M.A., AT THE UNIVERSITY PRESS, CAMBRIDGE


C37